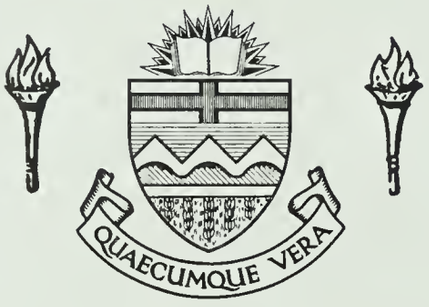

THE UNIVERSITY OF ALBERTA

# PURIFICATION OF UREASE FROM *PROTEUS MORGANII* BY AFFINITY CHROMATOGRAPHY

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Purification of Urease from *Proteus morganii* by Affinity Chromatography" submitted by Bassanio Loong Wong in partial fulfilment of the requirements for the degree of Master of Science.

## ABSTRACT

A simple, rapid procedure for the purification of urease (urea amidohydrolase; E.C. 3.5.1.5) from Proteus morganii by affinity chromatography was developed. Hydroxyurea, which was covalently linked via a long hydrocarbon side chain to beaded agarose, was used as the immobilized ligand to which urease could be adsorbed. Columns prepared from this substituted gel would specifically adsorb the urease from extracts of Proteus morganii, as well as the urease from jack bean meal. In both cases, the bound enzymes could be eluted from the adsorbent with 0.20 M PB (phosphate buffer with $10^{-3}$ M β-mercapto-ethanol) buffer, pH 4.6. The recovery of urease from cell-free extracts of P. morganii was usually greater than 200 per cent with a 170-fold purification by this single-step procedure. That 200 per cent of the urease activity present in the cell-free extract could be recovered indicated that either an endogenous urease inhibitor was removed or that activation of the enzyme occurred during the purification procedure. The specific activity and the yield of purified urease obtained by this technique was as high as, or higher than, those reported by other investigators who have used conventional, multi-step purification techniques. Catalytic staining of polyacrylamide gels after electrophoresis of the purified urease indicated that several isozymes of urease were present in the purified preparations.

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## LIST OF ABBREVIATIONS

EDTA - Disodium Ethylenediaminetetraacetate

PB - Phosphate buffer containing $10^{-3}$ β-mercaptoethanol

PEB - Phosphate buffer containing $10^{-3}$ EDTA and $10^{-3}$ β-mercaptoethanol

PPCNF - phenol-pentacyanonitrosylferrate reagent

TCS - Trypticase soy

TNBS - 2, 4, 6-trinitrobenzenesulfonate

TRIS - TRIS (hydroxymethyl) aminomethane

WSCD - 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (water-soluble carbodiimide).

# INTRODUCTION

Enzyme purifications which utilize conventional techniques are generally complex and time-consuming and the end-products of these multi-step procedures are often impure enzymes which have been isolated in low yields and with considerable loss of activity. These results are obtained primarily because classical techniques are directed towards separations based upon the physical and chemical properties of enzymes. Since these properties are relatively non-specific in nature, many problems are inherent in the use of these techniques for enzyme purification. The overlaps in the solubilities, sizes, shapes and charges of the numerous enzymes present, for example, in an extract of bacterial cells, almost guarantee that contaminating proteins will be present in any preparation containing a desired enzyme activity. Furthermore, as the number of steps in a purification sequence are increased, the residual contaminants in the enzyme preparation can be expected to have physical and chemical properties which are increasingly similar to those of the desired enzyme, so that the problem of separating an enzyme from residual contaminants can become even more complex during the later stages of a purification. As the enzyme itself is concentrated and purified, the residual contaminant with similar chemical and physical properties may also be concentrated and purified. For example, it has been found to be impossible to separate riboflavin phosphate phosphatase from highly purified flavokinase (ATP:riboflavin 5'-phosphotransferase; E.C. 2.7.1.26) with any of the conventional fractionation techniques

available and this has led to the belief that the phosphatase activity was an intrinsic property of flavokinase. This separation can easily be accomplished, however, by using affinity chromatography techniques, thus invalidating the earlier, erroneous conclusions (Arsenius et al., 1964).

Since, in conventional purification procedures which involve many fractionation and cut-off steps, it is a common practice to retain only fractions with high enzyme activities, the yield of an enzyme generally decreases with each additional step in the purification procedure and it is frequently necessary to begin with large quantities of starting material in order to obtain reasonable amounts of purified product. As well, the additional time spent as purification procedures are extended and the number of times that an enzyme is subjected to non-physiological conditions during the purification sequence, may also lead to enzyme inactivation. Once again, it should be emphasized that these difficulties which lead to low yields of enzyme activity are, for the most part, directly related to the use of fractionation techniques which are based upon differences in the physical and chemical properties of macromolecules.

If it were possible, however, to take advantage of the much more specialized biological properties of a given enzyme, it is obvious that few of the other proteins in a cell extract would also have those same, or similar, characteristics, and that a more efficient separation could be based upon those unique biological characteristics. Of course, as far as enzymes are concerned, the

most obvious of their specialized biological features, in addition to their antigen-antibody interactions, are their enzyme-substrate and enzyme-inhibitor affinities. Cuatrecasas et al. (1968) have recently developed an affinity chromatography technique which takes advantage of these types of biological specificities in the purification of macromolecules. This novel technique is both simple and rapid, while at the same time giving, under ideal conditions, an almost quantative yield of product with a very high specific activity and a minimum amount of impurities. Using the affinity chromatography technique, an enzyme in a crude extract can be highly purified by simply passing it through a gel column consisting of a cross-linked polymer to which is covalently bound a ligand that is capable of undergoing a strong association with the desired enzyme under the adsorption conditions employed. The enzyme for which the ligand is specific will thereby be retained on the column while those species in the crude extract which have little or no affinity for that ligand will pass through the column unretarded. The unadsorbed macromolecules can be washed out of the column with an appropriate buffer and the bound enzyme can then be eluted from the column. Elution can be achieved quite simply by manipulating conditions such as the acidity, the ionic strength or the temperature of the buffer (or combinations thereof) so that the affinity of the enzyme for the ligand is reduced. Alternatively, the addition of another, more potent, inhibitor or substrate of the enzyme can also, in principle, be used to elute the enzyme from the column.

There are three important components in an affinity chromatography system: first, the carrier, which is an insoluble matrix support; second, the ligand, which is covalently bound to the carrier and which should react specifically with that component whose purification is desired; and third, the component, in solution, which is to be purified. The carrier must be an insoluble, mechanically stable, and porous substance, which will not interact non-specifically with proteins or other macromolecules either before or after the ligand has been coupled to it. This type of carrier also differs from other conventional carriers, such as ion-exchange resins and activated charcoal, in that its chemically reactive groups must be such that a convenient and extensive covalent attachment of the specific ligand to the carrier can be carried out under a set of conditions which are mild enough so that there will be no disruption of the structural integrity of the carrier. The bond thus formed between the ligand and the carrier must also be stable under the conditions of adsorption and elution of the enzyme.

Of a variety of different polymeric carriers that have been used for affinity chromatography, the most commonly used substances have been polysaccharides (Mosbach, 1970; Mosbach and Mattiasson, 1970); cellulose (Axen et al., 1967; Poonian et al., 1971); starch and Sephadex (Axen et al., 1967); glass beads (Weetall, 1969; Weetall and Hersh, 1969), and beaded agarose (Porath et al., 1967; Cuatrecasas et al., 1968; Cuatrecasas and Wilchek, 1968; Pensky and Marshall, 1969; Cuatrecasas, 1970, 1970a; Poonian et al.,

1971; and Steers et al., 1971). Of these, beaded agarose, composed of alternating residues of D-galactose and 3, 6-anhydro-L-galactose, produced as described by Hjerten (1962), has been the carrier used most often in affinity chromatography, since it has the desired qualities of chemical inertness, mechanical stability, durability, and high porosity, in addition to possessing reactive groups which are suitable for the binding of a variety of ligands to the matrix. Although certain investigators prefer cross-linked polyacrylamide beads (Inman and Dintzis, 1969), since they possess a greater number of suitable reactive groups for ligand binding, most investigators seem to prefer beaded agarose because of its large pore size and consequently higher exclusion limits (Steers et al., 1971). However, depending upon the desired application of this technique, both carriers can be used to advantage.

An additional factor which, under some circumstances, may enter into the selection of the carrier matrix for an affinity chromatography system, is the flow rate of the resultant gel column. Again, beaded agarose has the desired quality of permitting a high flow rate, both before and after the binding of the ligand, which is retained even after adsorption of macromolecules to these columns. However, since the size of affinity columns can be kept very small and yet the column can still have a relatively high capacity for binding the desired enzyme, in most cases the flow rate of the column is not a significant factor in selecting the carrier matrix.

It has been observed, in some instances, that the adsorption of

macromolecules to the affinity columns was less than would be expected on the basis of amount of ligand bound to the carrier matrix (Cuatrecasas et al., 1968; Cuatrecasas, 1970; Cuatrecasas and Anfinsen, 1971, 1971a; Steers et al., 1971) and it was suggested that if the ligand were bound directly to the carrier matrix, steric hindrance, by the matrix, of the approach of a macromolecule to the ligand might account for the reduced adsorption. Cuatrecasas et al. (1968) tested this theory by intercalating hydrocarbon chains of various lengths between the ligand and the carrier matrix and found that as the distance between the ligand and the matrix was increased, the steric hindrance effects were reduced. It is now a standard procedure in affinity chromatography to bind ligands to the ends of long hydrocarbon chains, thus positioning the ligands at some distance from the carrier matrix in order to mimize or eliminate these effects entirely.

In all of the reported investigations wherein beaded agarose (usually Sepharose produced by Pharmacia, Uppsala, Sweden) has been used as the carrier, the side chain, to which the ligand was to be bound, was synthesized following activation of the agarose with cyanogen bromide under alkaline conditions as described by Cuatrecasas et al. (1968). This activation procedure has been adapted from general methods originally developed by Porath et al.(1967) and Axen et al.(1967) for coupling compounds containing primary aliphatic or aromatic amines to insoluble carbohydrate derivatives. The activated agarose will react easily with diamines of the type $NH_2\text{-}CH_2\text{-}(CH_2)_n\text{-}CH_2\text{-}NH_2$ to give

an ω-amino alkyl agarose derivative. This side chain may then be further extended by reacting this derivative with an anhydride, such as succinic anhydride, to give a succinyl-ω-aminoalkyl-agarose derivative. The length of the side chain will, of course, depend upon the exact nature of the diamines and anhydrides which are used, but at this stage, the free carboxyl group terminus of the side chain to which the ligand will be bound is usually far enough removed from the carrier matrix to minimize the effects of steric hindrance on the adsorption of macromolecules. A ligand can then be bound to the substituted agarose by taking advantage of the reactivity of the free terminal carboxyl group with the newly developed water-soluble carbodiimides (Cuatrecasas et al., 1968; Cuatrecasas, 1970; Cuatrecasas and Anfinsen, 1971, 1971a).

Since these carbodiimides can be used to effect a wide variety of reactions under relatively mild conditions, many different kinds of substances which can function as ligands can be covalently bound to the substituted agarose, thus increasing the versatility of the affinity chromatography technique. Using the carbodiimide activation technique, functional groups which can react with the substituted carrier include amino groups, carboxyl groups, sulfhydryl and hydroxyl groups, imidazole groups and phenolic groups (Silman and Katchalski, 1966; Cuatrecasas, 1970; Cuatrecasas and Anfinsen, 1971a).

As a result of the versatility of these reactions, a wide variety of substances may be used as ligands for affinity chromatography. For example, an antibody may be bound to the carrier matrix

for the purpose of purifying a specific antigen (Cuatrecasas, 1969; Cuatrecasas and Anfinsen, 1971), or an antigen may be used as a ligand for the purification of a specific antibody (Cuatrecasas, 1969; Omenn et al., 1969). Since antigen-antibody cross-reactions occur quite frequently, the homogeneity of products obtained when antigens or antibodies are used as ligands should be carefully investigated. Poonian et al. (1971) and Cozzarelli et al. (1967) have used nucleic acids and polynucleotides as ligands for the purification of complementary nucleic acids and polynucleotides. However, in this case, the specificity of the ligand is also less than ideal, since it is well known that non-homologous polynucleotides can aggregate to a certain extent when short, complementary nucleotide sequences are present.

If the ligand is an active enzyme, several alternatives are possible. For example, if the substrate of the enzyme is introduced into the column, along with the co-factors necessary for enzyme function, the product or products of the activity of that enzyme can be recovered from the column effluent (Mosbach, 1970, 1971; Mosbach and Mattiasson, 1970; and Cuatrecasas and Anfinsen, 1971). However, if substances necessary for the catalytic activity of the enzyme ligand are omitted, substrates, effectors, inhibitors and/or co-factors of that enzyme can, theoretically, be purified and concentrated at the same time. Alternatively, substrates and inhibitors of particular enzymes can be used as ligands for the purification of those enzymes (Cuatrecasas et al., 1968; Steers et al., 1971; Chan and Takahashi, 1969, and Chua and Bushuk, 1969). In these cases, the specificity of

the ligand is directed toward the catalytic site of the enzyme and, since enzyme catalysis is probably the most unique biological property exhibited by any of the macromolecules, it can be expected that enzyme preparations obtained in this way will contain a minimal amount of impurities.

It has been observed that only minor losses of enzyme activity occur when substrates, effectors or competitive inhibitors are used as ligands for enzyme purifications (Cuatrecasas et al. 1968; Steers et al., 1971; Wilchek and Gorecki, 1969; and Miller and Thompson, 1971). It was suggested that the interaction of the active site of an enzyme with the ligand might lead to stabilization, not only of the active site, but also of the entire tertiary structure of the enzyme, while it remains adsorbed to the ligand-matrix complex. Steers et al. (1971) have demonstrated that the β-galactosidase of E. coli can be stored adsorbed to an affinity column for several days and that it can then be eluted from the column with no loss of enzymatic activity having occurred during the storage interval. Active-site stabilization, by immobilized ligands, of inherently unstable enzymes for the purpose of storing purified preparations of those enzymes may well prove to be one of the more valuable applications of affinity chromatography in the future.

Ideally, the bound ligand should display a unique affinity for the subtance which is to be purified. As the specificity of the ligand is increased, the purity of the final product should also be increased, so that if the ligand reacts with one and only one

substance, then that substance should subsequently be eluted from the column in an essentially pure state. For example, Cuatrecasas et al. (1968) have been able to demonstrate that from a mixture of carboxypeptidase A (Peptidyl-L-amino-acid hydrolase; E.C. 3.4.2.1) and carboxypeptidase B(Peptidyl-L-lysine hydrolase; E.C. 3.4.2.2), carboxypeptidase A, but not carboxypeptidase B, could be adsorbed to a gel to which L-tyrosine-D-tryptophan, a specific inhibitor of carboxypeptidase A, had been bound as a ligand, and that a pure preparation of carboxypeptidase A could then be eluted from the column. However, if the ligand reacts strongly with the desired product, and at the same time reacts weakly with one or more other substances, then those other substances will very likely be present, at least as minor impurities, in the final preparation of the desired product. This point may be illustrated by the fact that preparations of wheat protease purified by Chua and Bushuk (1969) on a column which contained hemoglobin as the somewhat non-specific ligand, were found to be non-homogeneous upon analysis by polyacrylamide gel electrophoresis.

It is apparent, therefore, that the specificity of the ligand will determine the efficiency of the affinity chromatography purification procedure. Nevertheless, the usefulness of ligands which do not possess unique specificities should not be overlooked, since use of the affinity chromatographic technique for enzyme purification does not necessarily preclude the prior or subsequent application of conventional techniques to the purification problem. It should be emphasized that in those cases where single-step purification from

crude cell extracts by affinity chromatography cannot be achieved, this technique may still be of considerable value in augmenting the conventional purification techniques which must be employed. The value of affinity chromatography as a supplementary technique was demonstrated by Pensky and Marshall (1969) in their report on the purification of the thyroxine-binding globulin from human serum. Although these investigators were unable to obtain electrophoretically homogeneous preparations of this protein by affinity chromatography alone, such preparations could be obtained by a combined technique of affinity chromatograph followed by ion-exchange chromatography on DEAE-Sephadex columns (Pensky and Marshall, 1969).

Since binding of the ligand to the carrier involves a chemical reaction, both the carrier and the ligand must have reactive groups which are modifiable for that purpose. Moreover, as covalent binding of the ligand to the carrier cannot involve those functional groups of the ligand which are essential for its specificity, this factor is especially important when low molecular weight substrates and inhibitors are being considered as potential ligands. The masking of the important functional groups of these compounds which would result from involving them in the carrier-ligand bond could lead to an alteration of the specificity of the ligand, or, perhaps, to a marked reduction in its affinity for the desired product. Similarly, as many as possible of the reactive sites of enzymes, antibodies and antigens must also be free from involvement in the carrier-ligand bond whenever these macromolecules are employed as ligands, although at the

moment there are only a limited number of ways in which the binding of a macromolecule to a carrier matrix can be controlled.

That the technique of affinity chromatography has been and will continue to be extremely useful for the purification of biologically active substances is suggested by the increasing numbers of reports in the literature of the successful application of the basic principles of affinity chromatography to purification problems. Staphylococcal nuclease (Ribonucleate (deoxyribonucleate) 3'-nucleotidohydrolase; E.C. 3.1.4.7) has been purified in a single-step procedure with a yield of greater than 90 per cent on an activated agarose column containing 3'-(4-aminophenylphosphoryl)-deoxythymidine-5'-phosphate as the bound ligand (Cuatrecasas et al., 1968). Using hemoglobin as a ligand, Chua and Bushuk (1969) were able to obtain a 2-fold purification of wheat protease with a recovery of 90 per cent, when they chromatographed a preparation of this enzyme after stepwise fractionation with ammonium sulfate of a crude extract of wheat. Steers et al. (1971) also pre-treated extracts of E. coli with ammonium sulfate prior to the affinity chromatography step in their purification of β-galactosidase. Using p-aminophenyl-β-D-thiogalactopyranoside as the ligand, they were then able to obtain, in a single step, 100 per cent recovery of that enzyme in a preparation which gave a single band in polyacrylamide gel electrophoresis. Using D-tryptophan methyl ester as the ligand, Cuatrecasas et al. (1968) have purified α-chymotrypsin on an activated agarose column, while L-tyrosine has been used as a ligand for the purification of 3-deoxy-D-arabino-heptulosonate-7-

phosphate synthetase (Chan and Takahashi, 1969). Wilchek and Gorecki (1969) were able to purify ribonuclease A (ribonucleate pyrimidine-nucleotido-2'-transferase (cyclizing); E.C. 2.7.7.16) from bovine pancreas, using 5'-(4-aminophenylphosphoryl)-uridine-2 (3')-phosphate as the ligand.

Although most of the reports in the literature on the use of affinity chromatography have dealt with new methods for the purification of previously purified enzymes, in some cases novel purifications have been achieved. For example, the flavin-linked glycerol-3-phosphate dehydrogenase (L-glycerol-3-phosphate:cytochrome C oxidoreductase; E.C. 1.1.2.1) from mitochrondria, which could not be purified by conventional techniques, has now been purified 30-fold from rabbit muscle by Holohan et al. (1970) using 1-halodeoxy-DL-glycerol-3-phosphate as a ligand on an activated agarose column. Similarly, the separation of flavokinase (ATP:riboflavin 5'-phosphotransferase; E.C. 2.7.1.26) from contaminating riboflavin phosphate phosphatase activity, a separation which could not be achieved by conventional techniques, can now be effected by affinity chromatography when 7-cellulose-acetamido-6, 9-dimethylisoalloxazine is used as a ligand on CM-cellulose columns (Arsenius et al., 1964). A 120-fold purification of tyrosine aminotransferase (L-tyrosine:2-oxoglutamate aminotransferase; E.C. 2.6.1.5) from rat liver has also been obtained on activated agarose columns containing pyridoxamine phosphate as the ligand (Miller and Thompson, 1971). An additional 5-fold purification of this enzyme can be achieved if the active fractions from the affinity column are pooled

and subsequently chromatographed on a Sephadex G-200 column (Miller and Thompson, 1971).

Affinity chromatography has also been used in the purification of biologically active macromolecules other than enzymes. Insulin antibodies have been isolated from the immune sera of sheep by adsorption to activated Sepharose columns to which porcine insulin had been bound by its single lysine residue (Cuatrecasas, 1969). Using these purified insulin antibodies as ligands, Cuatrecasas and Anfinsen (1971) have since been able to isolate porcine insulin, but these preparations have generally been contaminated with other proteins which cross-react with the insulin antibodies. Other investigators have also reported that although specific antigens could be used effectively as ligands for the purification of antibodies, the use of purified antibodies as ligands for the isolation of specific antigens has been less successful (Wofsy and Burr, 1969; Wide and Porath, 1966; Weinstraub, 1970; and Givol et al., 1970). Nucleic acids have been isolated and purified by affinity chromatography on columns to which complementary nucleic acids have been bound as ligands and these same kinds of columns have also been used for the purification of nucleic acid polymerases, since those enzymes are also adsorbed by the appropriate nucleic acid ligands (Poonian et al., 1971; Cozarelli et al., 1967).

All of the above-cited purification procedures have certain features in common which illustrate the distinct advantage of using an affinity chromatography technique in preference to the classical

purification techniques. In many cases, single-step fractionations of cell-free extracts with affinity columns have resulted in the isolation of pure products. In other cases homogeneous products have been obtained when affinity chromatography has been augmented by the application of only one other conventional fractionation technique. However, regardless of whether or not supplementary procedures have been employed, the preparation of purified products from crude extracts has been accomplished in less than a single day, and in many cases within only a few hours. In addition, large amounts of enzyme have been purified on rather small gel columns within these time intervals. For example, using a column with the dimensions 1.5 x 22 cm, Steers *et al.* (1971) could purify up to 20 mg of *E. coli* β-galactosidase in a single run and Cuatrecasas *et al.* (1968) were able to purify 8 mg of staphylococcal nuclease in each experiment using a 0.5 x 5 cm gel column. Not only could large amounts of product be purified but, also, recovery of the desired product was routinely in the range of 90 - 100 per cent. As well, most of the ligand-matrix complexes that have been mentioned above are extremely stable, both structurally and chemically, and, unlike ion-exchange resins, need not be subjected to laborious regeneration procedures before they can be re-used. In fact, in many instances it is necessary only to wash the gel with distilled water prior to introducing a new sample of crude extract into the column.

These features perhaps most clearly illustrate the advantages of the affinity chromatography technique over the classical fractionation techniques for enzyme purification. However, other less

obvious advantages such as possible active-site stabilization of unstable enzymes, and economy of materials and of research time also serve to make affinity chromatography a very attractive research tool.

In addition to the purification of biologically active macromolecules on analytical and on preparative scales, affinity chromatography techniques can also be used in studies of enzyme behavior under conditions more closely approximating their natural microenvironment, since nearly all intracellular enzymes function *in vivo* either in a gel-like environment, as are the so-called soluble enzymes, or while adsorbed at an interface, as are membrane-bound enzymes. Investigations in this area by Silman and Katchalski (1966); Mosbach and Mattiasson (1970); Mosbach (1971); Cuatrecasas (1969a) and others, are made possible by studying the interactions of matrix-bound macromolecules. For example, Goldman *et al.* (1965) and Goldstein *et al.* (1970) have shown,respectively, that both matrix-bound papain and matrix-bound trypsin have pH optima which are dramatically different from the pH optima of those enzymes in dilute aqueous solutions. When hexokinase and glucose-6-phosphate dehydrogenase were bound to a single matrix, it could be shown (Mosbach and Mattiasson, 1970) that this system was twice as efficient as an aqueous solution of the two enzymes in the production of 6-phosphogluconolactone from glucose. These and similar studies with other matrix-bound enzymes have raised serious questions as to the validity of data obtained from earlier studies of enzymes which were carried out using dilute aqueous solution of those enzymes.

This investigation of the purification of urease (urea amidohydrolase, E.C. 3.5.1.5) by affinity chromatography arose out of the interest of Dr. F. L. Jackson, of this Department, in a study of the possible cross-reactions between ureases isolated from a variety of bacterial sources and their respective urease antibodies. Such a study requires not only that highly purified urease be available for the stimulation of specific antibody synthesis, but also that a rapid and efficient purification technique be available to facilitate the isolation of ureases from a wide variety of sources. The recently developed technique of affinity chromatography appeared to offer an ideal solution to both of these problems, and since urease is a well studied enzyme, this project also offered an excellent opportunity for a further evaluation of the usefulness of affinity chromatography in enzyme purifications. It was decided, therefore, to attempt to develop an affinity chromatography system which could be used for the purification of the urease of Proteus morganii.

## MATERIALS AND METHODS

1. Reagents: All chemicals were of reagent grade and were obtained from commercial suppliers.

Hydroxyurea, DL-α-amino-n-butyric acid hydroxamate and β-alanine hydroxamate hydrochloride were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A. 1-Ethyl-3-(3-Dimethylaminopropyl)-carbodiimide hydrochloride was obtained from Pierce Chemical Company, Rockford, Illinois, U.S.A. Cyanogen bromide was purchased from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. 'Sepharose 4B' was obtained from Pharmacia, Uppsala, Sweden. 'Affinose AF-202' was obtained from Control Atmosphere Incorporated, Anaheim, California, U.S.A. Sodium 2, 4, 6-trinitrobenzene sulfonate (TNBS) was the generous gift of Dr. S. Chu, formerly of the J. S. McEachern Laboratory, University of Alberta.

2. Methods

A. Organism and culturing conditions

i) *Proteus morganii* was obtained from the culture collection of the Department of Medical Bacteriology, University of Alberta, and was routinely grown on blood agar plates. Lyophilized stocks of this organism were also prepared for later use.

ii) Standard inoculum. Inocula were prepared from lyophilized stocks of *P. morganii* by subculturing the organism on each of 3 successive days on blood agar plates at 37°C. On the third day a single isolated colony was picked and inoculated into 50 ml of

of trypticase soy (TCS) broth and the broth was incubated for 18 hours at 37°C on a New Brunswick Reciprocating Incubator Shaker (New Brunswick Scientific Company, Inc., New Jersey, U.S.A.). The standard inoculum consisted of 10 ml of this 18-hour cell suspension per liter (final volume) of culture medium.

iii) Growth and harvest of the organism: A flask containing 890 ml of TCS broth was inoculated with the standard inoculum and incubated at 37°C with vigorous aeration for 18 hours. At that time, 100 ml of 40 per cent (w/v) urea, previously sterilized by Millipore filtration, were added to the culture and the incubation was continued as above for an additional 4 hours. The culture was then harvested by centrifugation at 13,200 x g for 10 minutes at 4°C in a Sorvall RC 2 B centrifuge (Ivan Sorvall Inc., Norwalk, Connecticut, U.S.A.). The packed cells were washed twice with distilled water and re-suspended in a total volume of 50 ml of distilled water.

B. Preparation of cell-free extract

Cell-free extract of P. morganii was prepared by immediately passing the suspension of washed cells through a Ribi cell fractionator (Ivan Sorvall, Inc., Norwalk, Connecticut, U.S.A.) operating at a pressure of 30,000 psi and at a temperature between 4°C and 10°C. The effluent from the fractionator was collected in a flask containing 5 ml of 0.20M phosphate buffer, pH 7.0, containing $10^{-2}$ M EDTA and $10^{-2}$M β-mercaptoethanol (0.20 M PEB buffer). The suspension was centrifuged at 27,000 x g for 10 minutes at 4°C. The resulting supernatant was

designated as"cell-free" extract. This extract was used without further treatment in chromatographic procedures. The cell-free extract was stored either at 4°C or at -30°C until required.

C. Standard curve for ammonium ion concentration

Ammonium ion concentrations were assayed by a modification of the technique of Van Slyke & Hillep (1933). A series of solutions containing between 0.025 µmole and 0.25 µmole of ammonium sulfate in 1.0 ml total volume of 0.02 M PEB buffer, pH 7.0, containing 0.05 M urea were prepared. To each of these solutions were added 2 ml of a solution containing 62.0 gm of reagent grade crystalline phenol and 0.2 gm of pentacyanonitrosylferrate per liter (PPCNF reagent). After mixing, 2.0 ml of alkaline hypochlorite reagent, containing 20.0 gm of sodium hydroxide and 43 ml of sodium hypochlorite (7.5 per cent available chlorine) per liter were added to each solution. After vigorous mixing, the solutions were then incubated in a water bath at 50°C for 6 minutes. Control tubes from which ammonium sulfate had been omitted were treated in a similar manner. The differences between the absorbances at 625 nm of the test mixtures and of the controls were determined with a Unicam S.P. 700 Recording Spectrophotometer (Unicam Instrument Ltd., Cambridge, England) and a standard curve relating the ammonium ion concentration to the differences in absorbance at 625 nm was constructed.

D. Urease assay

The reaction mixture contained an aliquot of enzyme solution

and 0.05 M urea in 0.02 M PEB buffer, pH 7.0, in a final volume of 1.0 ml. The reaction mixture was incubated at 25°C for 2 minutes. Reagent blanks from which the enzyme was omitted were also prepared and treated in a similar manner. The reaction was terminated at 2 minutes by the addition of 2.0 ml PPCNF reagent. After the addition of alkaline hypochlorite reagent, this mixture was incubated at 50°C for 6 minutes, and the ammonium ion which was released as a result of the ureolytic activity of the urease was determined by measuring the differences in the absorbances at 625 nm of the reaction mixtures and the controls and referring to the previously prepared standard curve. Enzyme activity was expressed as μmoles of urea hydrolyzed per mg of protein per minute at 25°C.

E. Protein determination

Soluble protein was determined by the method of Lowry et al. (1951) using electrophoretically pure crystalline bovine serum albumin as the reference protein. The protein content of column effluents was followed by measuring the absorbance at 280 nm of the eluted fractions.

F. Preparation of the ligand-matrix complex

Affinose AF 202, a succinylated derivative of beaded amino-alkyl agarose, was washed with 20 volumes of 0.1 M sodium chloride to remove the sodium azide originally included in the gel suspension as a bacteriostatic agent. The gel was then washed with distilled water

until sodium chloride could no longer be detected in the column effluent. The washed gel was resuspended in a small volume of distilled water and the acidity of the gel suspension was adjusted to pH 5.0 with 0.1 N HCl. Hydroxyurea (4.36 mg/ml of packed gel) was dissolved in a minimum volume of distilled water and added to the gel suspension. The acidity of the mixture was then adjusted to pH 4.6 with 0.1N HCl. A water-soluble carbodiimide (WSCD; 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide), dissolved in a minimum volume of distilled water (26.4 mg WSCD/ml of packed gel) was added dropwise over a 5-minute period while the mixture was mechanically stirred. The total volume of the reaction mixture was not permitted to exceed 2 times the packed volume of gel in order to minimize the dilution of the reagents involved in the substitution reaction. The mixture was then incubated at room temperature for 20 hours. At intervals during that period, portions of the reaction mixture were removed in order to determine the extent of binding of hydroxyurea to the gel using the 2, 4, 6-Trinitrobenzene sulfonate-borate color test described below. After 20 hours, the gel was packed in a glass chromatography column and washed extensively with distilled water (6 liter/10 ml packed gel). In a similar manner, a ligand-matrix complex was prepared from Affinose AF-202 using β-alanine hydroxamate hydrochloride (5.9 μmole/ml packed gel) as the ligand rather than hydroxyurea.

A ligand-matrix complex derived from Sepharose 4B was also prepared. An aminoalkyl side chain derived from ethylene diamine was attached to cyanogen bromide-activated beaded Sepharose 4B, using the

methods described by Cuatrecasas (1970). The free amino group of this side chain was then reacted with succinic anhydride as described by Cuatrecasas (1970), in order to form a succinylated aminoalkyl derivative of Sepharose 4B analogous to Affinose AF 202. Hydroxyurea could then be bound to this matrix in the same manner as described above for the binding of hydroxyurea to Affinose AF 202.

G. Test for substitution of gel (TNBS-borate color test).

The qualitative color test which reflects the degree of binding of ligand to an agarose matrix, as described by Cuatrecasas (1970), was modified slightly for convenience. A 2.0 ml portion of the reacting gel was packed into a small glass column prepared by plugging the tapered end of a Pasteur pipette with glass wool and the small column was then washed extensively with distilled water. After this washing, 2 ml of saturated sodium borate solution, to which was added 6 drops of 3 per cent (w/v) TNBS, was passed into the gel column and allowed to react with the gel at room temperature for 2 hours. The development of a distinctive color in the gel column was noted.

H. Qualitative test for hydroxyurea

The test, adapted from Davidson (1940), involved the addition of a drop of 10 per cent ferric chloride to the test solution. A purple-blue color indicated the presence of hydroxyurea.

I. Affinity chromatography

The washed ligand-matrix complex was packed in glass

chromatography columns and equilibrated with 0.02 M phosphate buffer, pH 7.0, containing $10^{-3}$ M β-mercaptoethanol (0.02 M PB buffer) immediately before chromatography. Cell-free extract which was known to contain urease activity was passed through the gel column and the gel was then washed with 0.02 M PB buffer, pH 7.0, under a constant hydrostatic pressure head of 50 cm. Fractions containing 3.0 - 3.3 ml of column effluent were collected using an LKB Ultravac Automatic Fraction Collector, Type 7000 (LKB-Produkter AB, Stockholm-Bromma 1, Sweden). The column was washed with this buffer until the absorbance of the effluent, as measured at 280 nm, was less than 0.01. At that point, the wash buffer was replaced with an elution buffer containing 0.20 PB buffer, pH 4.6. Elution was continued until the absorption of the column effluent was again less than 0.01, as measured at 280 nm. All fractions were assayed for urease activity as described above. After elution of urease activity, the column was washed with 0.40 M $KH_2PO_4$, containing $10^{-3}$ M β-mercaptoethanol pH 4.6 (80 ml/ml of packed gel), and then extensively washed with distilled water. After this wash, the column could again be equilibrated with 0.02 M PB buffer, pH 7.0, and re-used. Fractions containing urease activity were pooled and stored at 4°C.

J. Polyacrylamide (disc) gel electrophoresis

Samples were dialyzed against 1300 volumes of $10^{-3}$ M β-mercaptoethanol overnight at 4°C with one change of solution. The dialyzed material was concentrated by lyophilization in a New Brunswick Cryolizer, Model B 65 (New Brunswick Scientific Company Inc., New Brunswick, N.J.,

U.S.A.), and then redissolving in a minimum amount of distilled water.

Polyacrylamide gel electrophoresis of the proteins was carried out in 9 x 65 mm or 9 x 90 mm tubes according to the procedures of Davis (1964) except that no sample gels were used and the sample was introduced onto the spacer gel in a solution of 20 per cent sucrose. The spacer gels were buffered at pH 6.7 with 0.06 M Tris-HCl and the separating gels were buffered at pH 8.9 with 0.37 M Tris-HCl. The tank buffer was 0.05 M Tris-glycine, pH 8. Electrophoresis proceeded for 4 hours at 4°C, using a current of 2 mA per gel tube. Protein bands were then stained with 1 per cent Amido black in 7 per cent acetic acid. De-staining was carried out either electrophoretically or by diffusion in 7 per cent acetic acid and the gels were photographed. Bands which contained urease activity were stained catalytically as described by Fishbein (1969; personal communication) as follows: after electrophoresis the gel was equilibrated with 0.05 M citrate buffer, pH 6.0, by gently rocking the gel in the buffer at 25°C. The buffer was changed frequently until the pH of the buffer remained at pH 6.0 for at least 2 hours. The gel was then placed in a solution containing distilled water: 0.5 per cent paranitroblue tetrazolium: 0.50 M citrate buffer, pH 6.0: and 0.1 M urea (19:2.5: 1.8: 2.6 (v/v)), to which was added 7 mg of dithiothreitol, and allowed to react for 8 hours at 25°C. Catalysis was then stopped by immersing the gel in 1.0 M HCl for 1 hour. Since the gels shrink quite rapidly when treated in this manner, they were washed with distilled water and photographed as quickly as possible.

## RESULTS AND DISCUSSION

### Urease preparations

When suspensions of *P. morganii* were disrupted in the Ribi fractionator, greater than 90 per cent breakage was routinely observed as determined by phase-contrast microscopy and viable cell counts. The cell-free extract obtained from a 2-liter culture of *P. morganii* usually contained about 8 mg of protein/ml and the urease activity in the cell-free extract of cultures induced for four hours with 4 per cent (w/v) urea was in the order of 15 I.U./mg of protein. If the cultures were not induced with urea, the cell-free extract contained only about 7 IU of urease/mg of protein. Cells which had been induced for 4 hours with urea were routinely used as a source of urease in the following experiments.

The urease activity in the cell-free extract was found to be relatively stable, since the preparations could be stored in 0.02 M PEB buffer pH 7.0, for a period of 10 days at 0°C with less than 20 per cent loss of activity. Purified jack bean urease (Sigma Chemicals) containing 300 IU of urease activity per mg of protein was used in many of the preliminary experiments in order to establish the conditions necessary for the adsorption of urease to the substituted gel and for the subsequent elution of the enzyme. The rationale behind the use of jack bean urease rather than of *P. morganii* urease in the preliminary experiments was that the commercial preparation was a very convenient source of the desired enzyme. In addition, however,

since the objective of this investigation was to prepare an affinity system which could be used to purify urease from a wide variety of sources, it was suggested that if a ligand-matrix complex could be devised which could be used to purify both jack bean urease and the urease from P. morganii, then that ligand-matrix complex might also be expected to be useful in the purification of ureases from other sources as well.

A stock solution containing 1300 IU of urease/ml was prepared by dissolving commercial jack bean urease (4.3 mg/ml) in 0.02 M PEB buffer pH 7.0 and this was diluted with the same buffer before being used in the chromatographic procedures.

Preparation of the ligand-matrix complex

Hydroxyurea ($NH_2$-CO-NHOH) was ultimately selected as the ligand over a variety of other potentially useful compounds for a number of reasons. It had been reported by Kobashi et al. (1962) and Hase and Kobashi (1967) that many hydroxamic acids, and in particular the long-chain aliphatic hydroxamic acids, were potent inhibitors of both jack bean urease and the ureases from Proteus species, and it was believed that the carbodiimide-mediated reaction between the free carboxyl group on the side chain of the Affinose AF 202 gel and the amino group of the added hydroxyurea would likely result in the formation of a hydroxamic acid-substituted side chain which, in many respects, would be very similar to a long-chain aliphatic hydroxamic acid (Cuatrecasas, 1970; Mosbach, 1971). The proposed reaction

mechanism is illustrated in Fig. 1. The hydroxamic acid-substituted side chain, therefore, might be expected to function as an inhibitor of urease, and as an inhibitor immobilized by being covalently linked to the gel, it might also be expected, under appropriate conditions, to retain the urease within the gel column. Another consideration which suggested that hydroxyurea might effectively be used as the ligand was the fact that hydroxyurea itself had been reported by Fishbein (1965a) to be an inhibitor of urease activity. In addition, hydroxyurea is relatively inexpensive, readily available, and highly soluble, in contrast with many of the other compounds which were considered. These alternative compounds were generally β- or ω-amino-, aliphatic hydroxamic acids, since a free amino group is required for the carbodiimide-mediated reaction with the free side-chain carboxyl group of the Affinose AF-202 gel. In general, however, these compounds were expensive, difficult to obtain and, for the post part, were very insoluble in aqueous solutions and therefore appeared to be unsuitable alternatives to hydroxyurea.

Using Affinose AF-202 as the carrier matrix, optimum binding of the ligand was achieved when the gel was reacted with $5.74 \times 10^{-5}$ moles of hydroxyurea and $1.38 \times 10^{-4}$ moles of water-soluble 1-Ethyl-3-(3-Dimethylaminopropyl) carbodiimide (WSCD) per ml of packed gel for 20 hours at room temperature. When the acidity of this reaction mixture was monitored, an initial drop from pH 4.6 to pH 4.2 was noticed upon the first addition of WSCD. This was followed by a continued rise as additional WSCD was added, until, after all of the

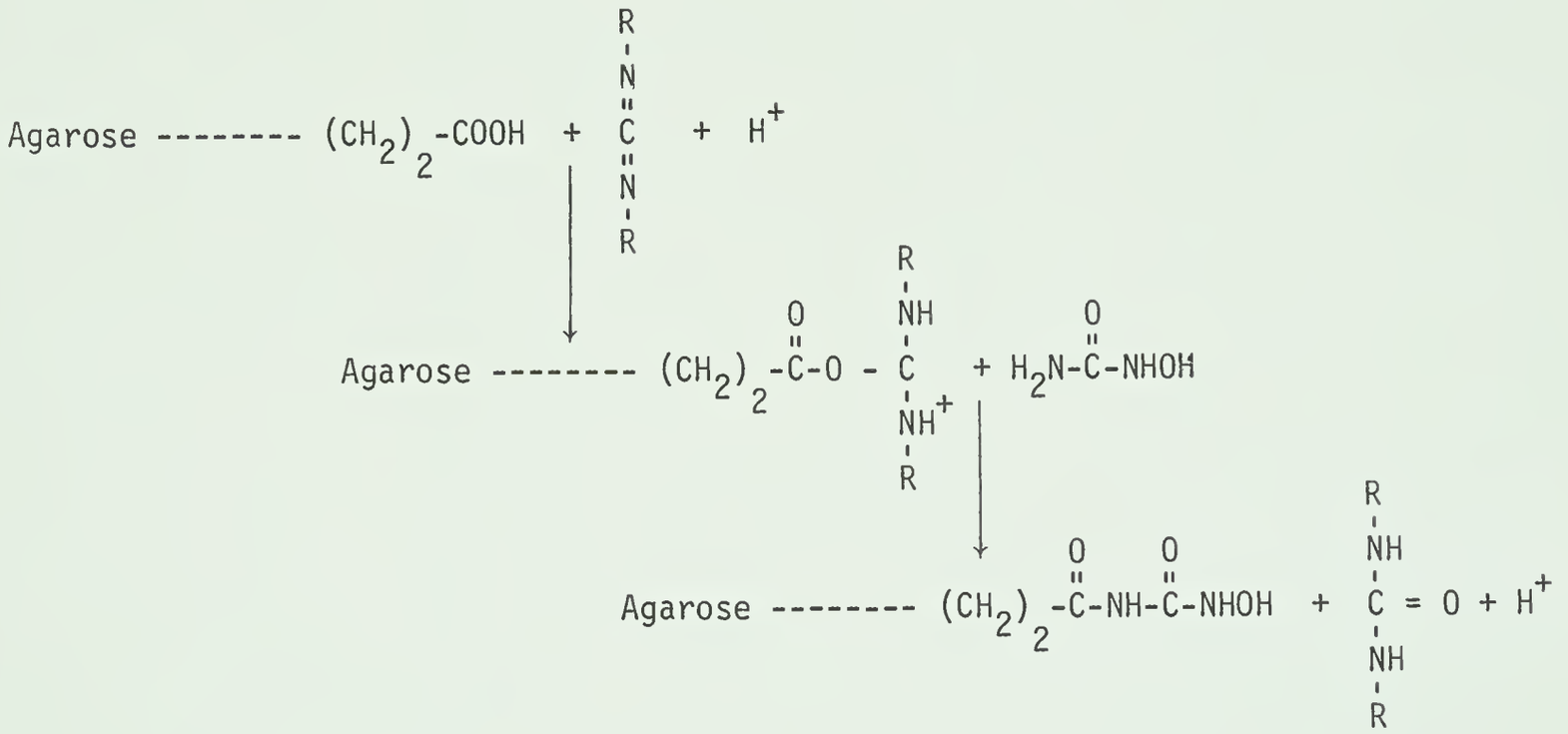
Agarose -------- (CH2)2-COOH + R-N=C=N-R + H+
Agarose -------- (CH2)2-C(=O)-O-C(=NH+-R)(NH-R) + H2N-C(=O)-NHOH
Agarose -------- (CH2)2-C(=O)-NH-C(=O)-NHOH + C(=O)(NH-R)(NH-R) + H+

FIG. 1. Reaction mechanism involved in the formation of hydroxyurea-Affinose.

(This mechanism was proposed by Mosbach (1971) and Cuatrecasas and Anfinsen (1971).)

Agarose -------- $(CH_2)_2$-COOH Affinose AF-202

R-N=C=N-R 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide

$H_2N$-C(=O)-NHOH Hydroxyurea

reagent was added, the acidity of the reaction mixture stabilized at pH 6.5 to 6.8. After 20 hours at 25°C the final pH of the reaction mixture was usually found to be between pH 7.0 and 7.2. During the course of the reaction, the reaction mixture changed color from white to pale yellow, while at the same time, the characteristic odor of an ester could be detected. A gas was also evolved from the reaction mixture, but no attempts were made to characterize either the colored component, or the ester, or the gas.

From time to time during the course of the reaction, portions of the reaction gel were poured into Pasteur pipettes plugged with glass wool, and the resulting columns of gel were washed extensively with distilled water until the effluents were negative to $FeCl_3$ (indicating that hydroxyurea was no longer present). Subsequently, a solution of saturated sodium borate to which was added 3 per cent (w/v) TNBS (3 drops/ml of sodium borate) was passed into the column of washed gel. In those cases where the carbodiimide-mediated substitution reaction was taking place, a distinctive orange color developed in the column of gel, the intensity of this color being greater in gel samples that had reacted with WSCD and hydroxyurea for longer periods of time, although the rates of development of color, as well as the color intensities, varied from one gel preparation to another. It was observed, however, that although the rapid appearance of intense orange color always indicated that a particular gel preparation would have a relatively high capacity for urease

binding, those preparations in which color development was slow and in which the final color was much less intense, also possessed a significant capacity for binding urease. This could be shown directly by measuring the amount of urease which could subsequently be eluted from those columns. Therefore, although the degree of substitution of the gel with hydroxyurea may be reflected by the TNBS-borate color test, it was concluded that a more accurate assessment could be made by measuring the amount of urease retained by the ligand-matrix complex.

Washing these gels with water or with 0.1 M EDTA removed the orange color, but the readministration of TNBS-borate reagent rapidly regenerated this color. This finding suggested that the orange color developed as a result of the reaction of this reagent with hydroxyurea which was covalently bound to the carrier matrix and that it did not result from a reaction with amino groups on unbound molecules within the gel bed, since those molecules would be removed by the washing procedure.

Since unsubstituted Affinose AF-202 did not give an orange color reaction with the TNBS-borate reagent, the appearance of this color in gels which had been reacted with WSCD and hydroxyurea indicated that the free carboxyl groups of the Affinose AF-202 were being masked by residues containing amino groups. This in turn suggested that the hydroxyurea was being bound to the side-chains of the gel, by way of the free carboxyl groups and that it was possible that a terminal hydroxamic acid derivative was being formed. It has

not been possible to prove conclusively that the side chain terminus of the substituted gel is, in fact, a hydroxamic acid since the tests for hydroxamic acids which are now available require that soluble compounds be present so that multimolecular aggregates can be found which exhibit a distinctive color (Davidson, 1940). In a substituted gel, in which the mobility of the reactive groups is limited these aggregates cannot be formed. However, the reaction mechanism which has been postulated to occur between carboxyl and amino groups when mediated by carbodiimides does suggest that a hydroxamic acid terminus would result from the reaction described above.

A ligand-matrix complex using Sepharose-4B as the carrier was also prepared and an attempt was made to use this complex for the affinity chromatography purification of urease. Sepharose-4B was activated with cyanogen bromide and a side chain was synthesized using ethylene diamine and succinic anhydride according to the procedures of Cuatrecasas (1970) as outlined in Materials and Methods. Hydroxyurea (57.5 μmoles/ml packed gel) was again used as the ligand and was coupled to the free carboxyl terminus of the carrier using WSCD under the same conditions as were described for the substitution of Affinose AF-202. When the degree of substitution of this gel with hydroxyurea was tested with the TNBS-borate reagent, an extremely intense orange color appeared very rapidly, indicating that there might be a much more extensive substitution of this gel, relative to the substitution of Affinose AF-202 which was achieved under the same conditions. When P. morganii cell-free extract was chromatographed on the substituted

Sepharose-4B column using the same conditions as described below for use with the substituted Affinose AF-202 gel, the chromatographic profiles, as far as the elution of protein from the columns was concerned (Fig. 2), were similar to those obtained with substituted Affinose AF-202 except that a larger second protein peak was eluted from the column. However, when effluent fractions were assayed for urease activity, no enzyme activity could be detected (Fig. 2). Attempts to obtain active urease preparations after chromatography on these columns following additional extensive washing of the substituted gels, as well as after altering the concentration of the hydroxyurea used in the coupling reaction or after using an alternative ligand (DL-α-amino-η-butyric acid hydroxamate 5.9 μmoles/ml of packed gel) were unsuccessful. Although this gel appeared to possess a potentially greater capacity for binding urease, as indicated by the TNBS-borate color test as well as by the amount of protein which could be eluted from the column after the buffer change, the problem of obtaining an active preparation of urease from those columns has not, as yet, been solved, and it has been necessary to carry out subsequent experiments using the substituted Affinose AF-202 columns.

Affinity chromatography

Preliminary experiments indicated that when a solution of jack bean urease dissolved in 0.02 M PB buffer, pH 7.0 was passed through a column of hydroxyurea-substituted Affinose Af-202, urease activity was retained on the column even though the column was washed

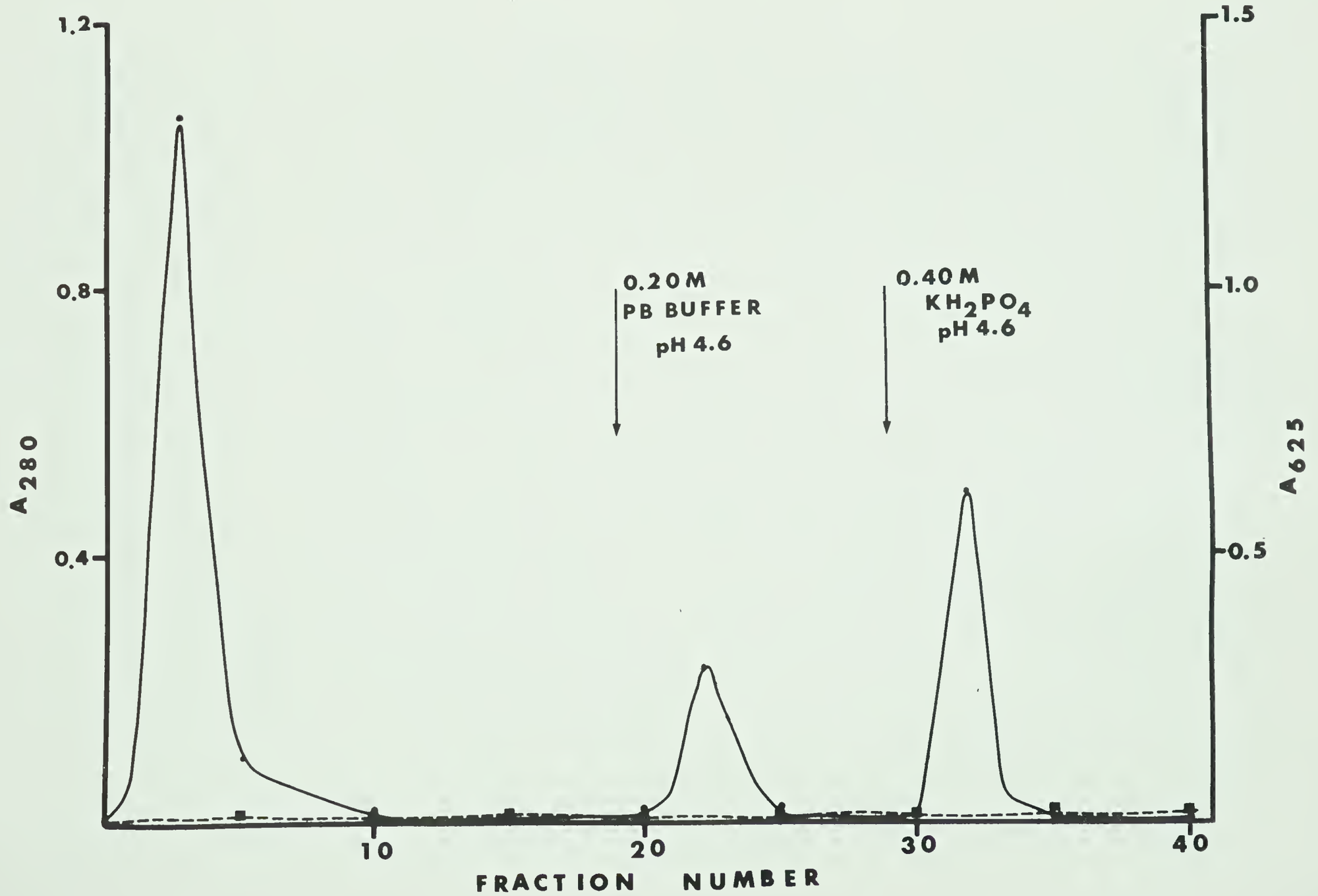
1.2
0.8
0.4
1.5
1.0
0.5
$A_{280}$
$A_{625}$
0.20 M
PB BUFFER
pH 4.6
0.40 M
$KH_2PO_4$
pH 4.6
10
20
30
40
FRACTION NUMBER

FIG. 2. Affinity chromatography of cell-free extract of *P. morganii* on hydroxyurea-Sepharose column.

1.0 ml of cell-free extract in 0.02 M PB buffer pH 7.0 was introduced into, and chromatographed on a 1.5 x 6.0 cm affinity column in which hydroxyurea was bound to activated Sepharose 4B, at a constant flow rate of 50 ml/hr. 3.3-ml fractions were collected. Protein was expressed as absorbance at 280 nm and urease activity as the change in absorbance at 625 nm produced by 0.5 ml of the fraction in the assay system described in Materials and Methods. 0.40 M $KH_2PO_4$ pH 4.6 contains $10^{-3}$ β-mercaptoethanol.

Absorbance at 280 nm. ——●——

Change in absorbance at 625 nm. ■- - - - - - - -■

extensively with additional buffer (Fig. 3). However, the retained urease could subsequently be eluted from the column by simultaneously increasing the ionic strength and lowering the pH of the buffer (Fig. 3), as described below.

If hydroxyurea was omitted from the gel-substitution reaction mixture, no urease activity was retained on the gel column (Fig. 4 (a)). Similarly, if WSCD was omitted from the reaction mixture, again no urease activity was retained on the column (Fig. 4 (b)). In both cases all of the added urease activity emerged with the first protein peak and was completely removed from the column by the washing procedure. Only when both hydroxyurea and WSCD were included in the reaction micture with Affinose AF-202 did the resultant gel possess the ability to adsorb urease activity and the adsorbed urease could then be eluted from the gel only when the appropriate elution buffer was passed through the column (Fig. 4 (c)).

Conditions for the elution of urease

A small volume of the cell-free extract from P. morganii was passed into a 1.5 x 4.5 cm column of hydroxyurea-substituted Affinose AF-202 and the column was washed with 0.02 M PB buffer, pH 7.0, until the effluent was free of protein, as determined by measuring the absorbance of the effluent at 280 nm. An attempt was then made to elute urease activity from the column with a pH gradient which increased in acidity from pH 7.0 to pH 3.75. The gradient was formed in an open, 2-chambered gradient-forming apparatus with 35 ml of 0.02 M

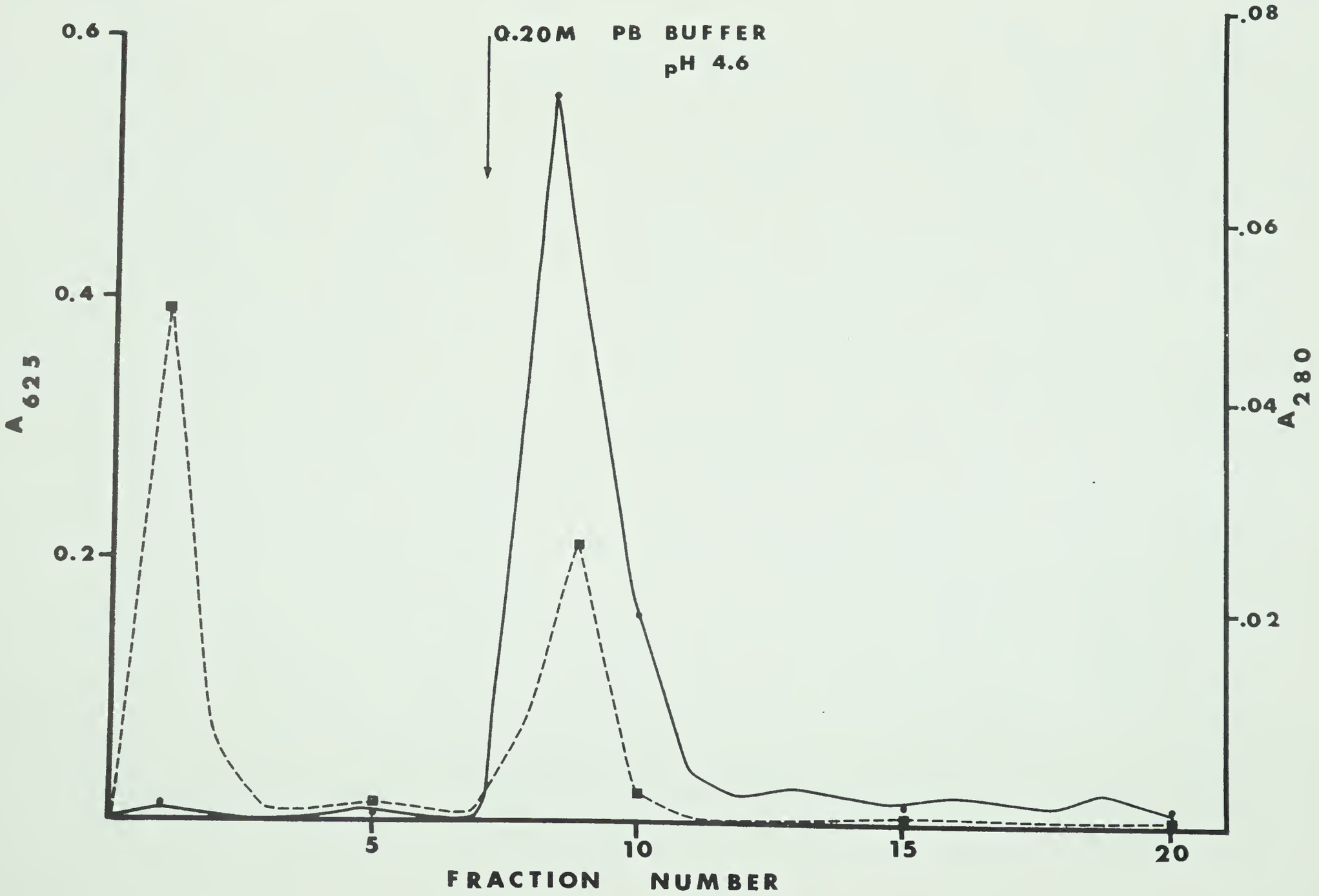
0.20M PB BUFFER
pH 4.6
0.6
0.4
0.2
A625
.08
.06
.04
.02
A280
5
10
15
20
FRACTION NUMBER

FIG. 3. Affinity chromatography of jack bean urease on hydroxyurea-Affinose column.

0.05 mg of jack bean urease in 0.1 ml of 0.02 M PEB buffer pH 7.0 was added to the column (0.9 x 1.5 cm) and chromatographed as described in Materials and Methods. 3-ml fractions were collected at a constant flow rate of 96 ml per hour. Protein concentration was expressed as absorbance at 280 nm and enzyme activity as the change in absorbance at 625 nm produced by 100 λ of the fraction in the assay system under the conditions described in Materials and Methods.

0.40 M $KH_2PO_4$ pH 4.6 contains $10^{-3}$ M β-mercaptoethanol.

Absorbance at 280 nm. ■----------■

Change in absorbance at 625 nm. ●——————●

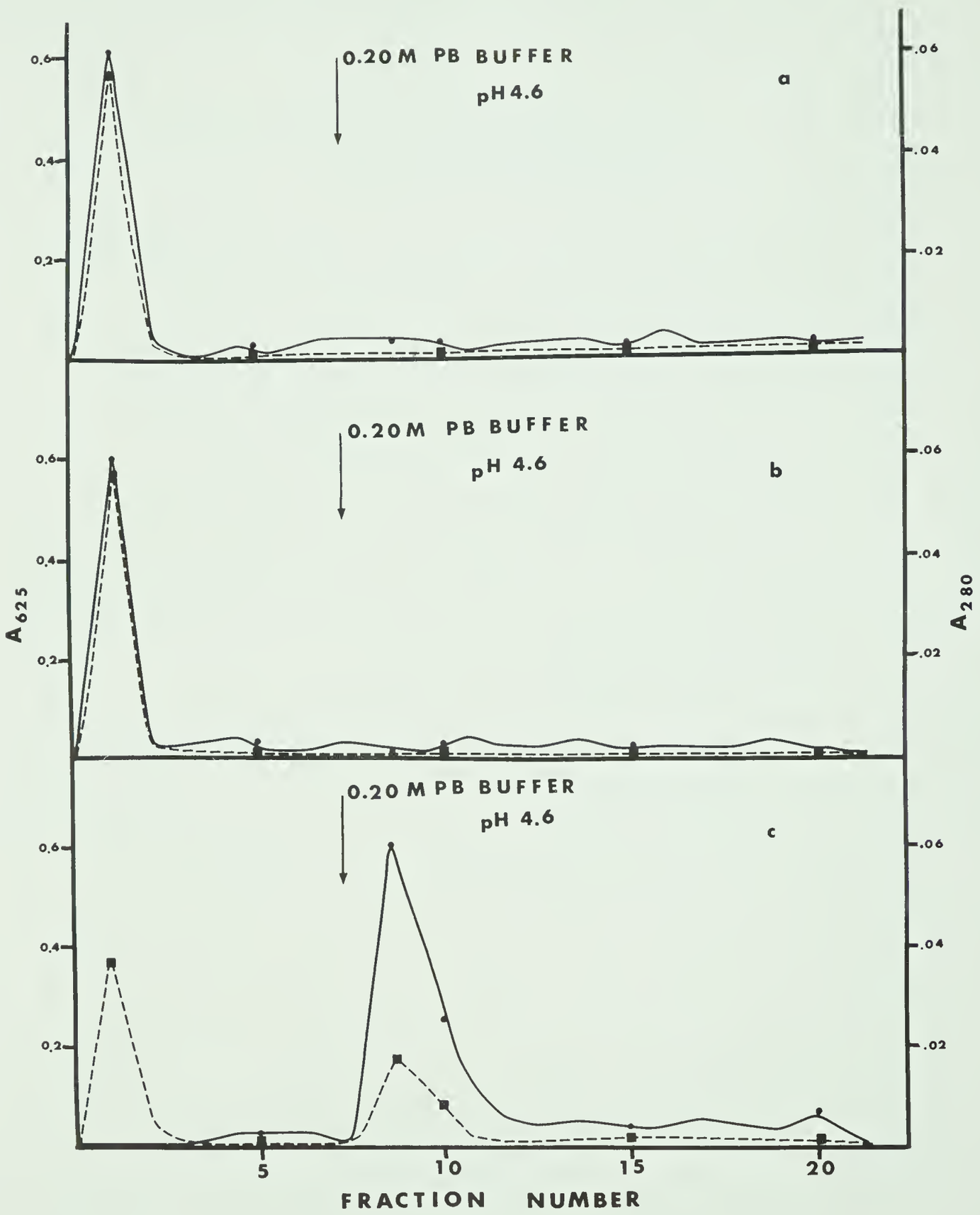
0.20 M PB BUFFER
pH 4.6
a
0.20 M PB BUFFER
pH 4.6
b
0.20 M PB BUFFER
pH 4.6
c
A625
A280
0.6
0.4
0.2
.06
.04
.02
5
10
15
20
FRACTION NUMBER

FIG. 4. Substitution of Affinose AF-202 with Hydroxyurea.

Jack bean urease was chromatographed as previously described on 0.9 x 1.5 cm column prepared as follows:

a. Hydroxyurea was omitted from the reaction mixture in the preparation of the 'ligand-matrix' complex.

b. Carbodiimide was omitted from the reaction mixture in the preparation of the 'ligand-matrix' complex.

c. Complete reaction mixture was present in the preparation of the 'ligand-matrix' complex.

Protein concentration was expressed as absorbance at 280 nm and enzyme activity as the change in absorbance at 625 nm produced by 100 λ of a fraction added to the assay system described in Materials and Methods.

Absorbance at 280 nm. 

Change in absorbance at 625 nm. 

PB buffer, pH 3.75, in the reservoir. The acidity of the column effluent was monitored and when the acidity reached pH 3.75 the gradient was discontinued and an additional 10 ml of 0.02 M PB buffer, pH 3.75, was passed into the gel column. As can be seen in Fig. 5, this procedure did not result in the elution of urease activity from the column.

The column was then washed with distilled water and re-equilibrated with 0.02 M PB buffer, pH 7.0, and a gradient of PB buffer which increased in ionic strength from 0.02 M to 0.40 M at pH 7.0 was passed through the column. As can be seen in Fig. 5, this treatment did result in the elution of urease activity from the column. However, at this point there was no assurance that all of the bound urease had been eluted from the gel. Therefore, the column was again washed with distilled water and equilibrated with 0.02 M PB buffer, pH 7.0, and eluted first with 30 ml of 0.20 M PB buffer, pH 4.6, and then with 30 ml of 0.40 M $KH_2PO_4$ containing $10^{-3}$ M β-mercaptoethanol, pH 4.6. The results of these experiments, also illustrated in Fig. 5, indicate that elution of urease activity by the concentration gradient at pH 7.0 was not complete, since additional urease activity could be eluted from the column when 0.20 M PB buffer, pH 4.6, was passed through the gel. However, subsequent washing of the column with 0.40 M $KH_2PO_4$, containing $10^{-3}$ M β-mercaptoethanol, pH 4.6, failed to elute additional protein or urease activity from the gel. As a result of these experiments it was concluded that 0.20 M PB buffer, pH 4.6, was an adequate buffer for the elution of bound urease from the hydroxy-urea-

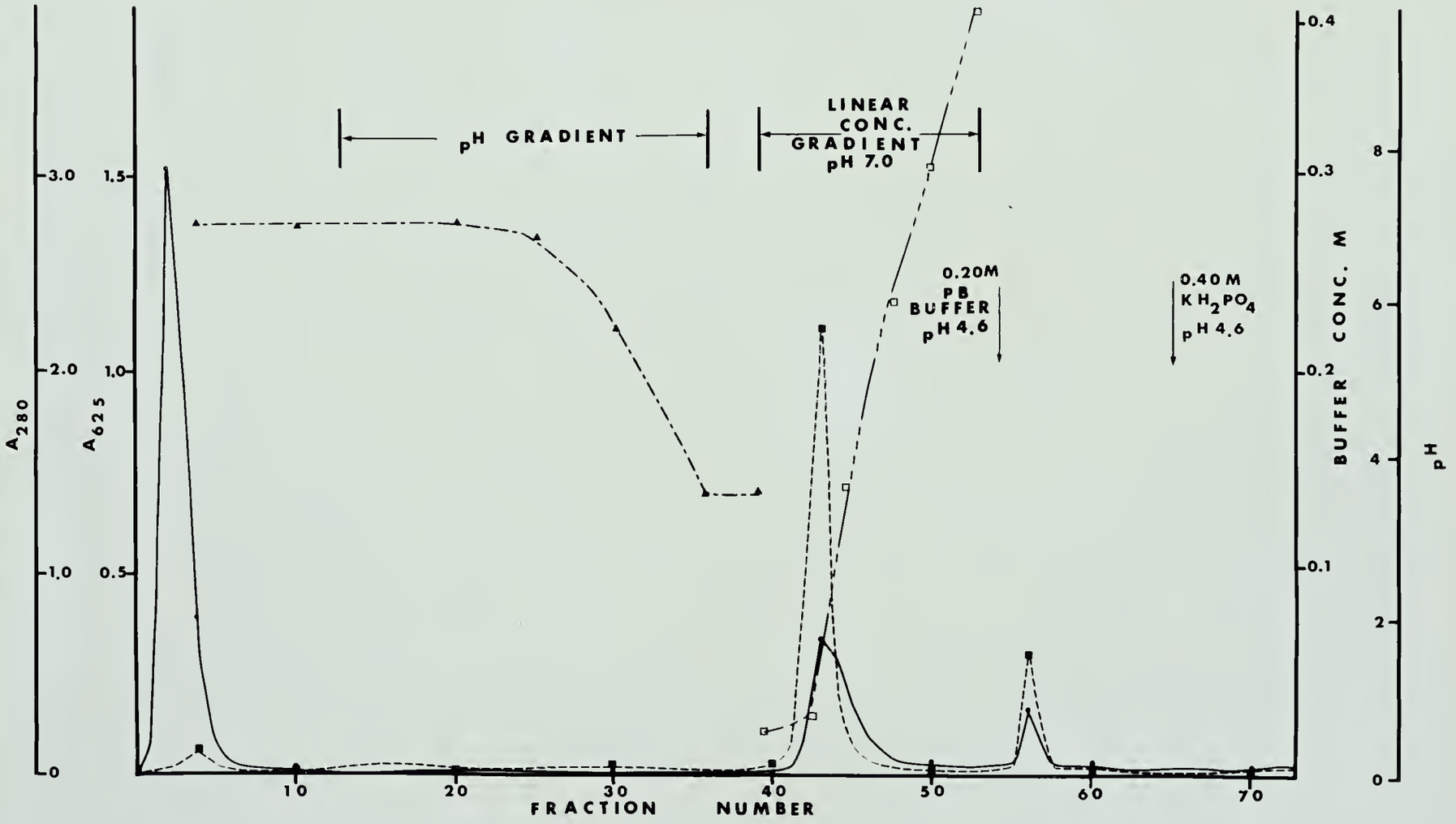
pH GRADIENT
LINEAR CONC. GRADIENT pH 7.0
0.20M PB BUFFER pH 4.6
0.40 M $KH_2PO_4$ pH 4.6
$A_{280}$
$A_{625}$
BUFFER CONC. M
pH
FRACTION NUMBER
3.0
2.0
1.0
0
1.5
1.0
0.5
0.4
0.3
0.2
0.1
8
6
4
2
0
10
20
30
40
50
60
70

FIG. 5. Buffer composition for elution of adsorbed urease.

0.5 ml of cell-free extract in 0.02 M PB buffer, pH 7.0, was added to a hydroxyurea-Affinose column and washed with the same buffer until fraction 13. It was then eluted with a pH gradient of the same buffer from pH 7.15 to pH 3.75 until fraction 35. 10 ml of this buffer pH 3.75 was then used to wash the gel. After washing with distilled water and 0.02 M PB buffer pH 7.0, the column was eluted with a linear concentration gradient of 0.02 M - 0.40 M PB buffer pH 7.0. 0.20 M PB buffer pH 4.6 was added at fraction 55 after washing and equilibration with 0.02 M PB buffer pH 7.0 and 0.40 M $KH_2PO_4$ pH 4.6 at fraction 65. Flow rate of the wash was 50 ml/hr and that of linear gradients 35 - 50 ml/hr. 3-ml fractions were collected and assayed as previously described. Protein was expressed as the absorbance at 280 nm and urease activity as the change in absorbance at 625 nm produced by 5 λ of the fraction in the assay system as described in Materials and Methods.

Absorbance at 280 nm. ●——————●

Change in absorbance at 625 nm. ■- - - - - - - - - - ■

pH ▲- - —- - —- - —- - ▲

Buffer concentration □- - - ——— - □

substituted Affinose AF-202 columns, and that buffer was therefore used as the elution buffer in subsequent experiments.

The use of an acidic elution buffer was based upon the observations of Kobashi and Hase (1966), who reported that urease-hydroxamic acid complexes tended to dissociate below pH 5.2 to 6.0. Therefore, since it was necessary to determine whether residual urease was still bound to the affinity column after elution with the concentration gradient at pH 7.0, it was suggested that a buffer which had a moderately high acidity as well as a high ionic strength might be useful. The results of the experiments reported above indicate that this reasoning was valid, since complete elution of bound urease could be accomplished with 0.2 M PB buffer, at pH 4.6.

Although it was reported by Larson and Kallio (1954) and by Hase and Kobashi (1967) that the ureases from Bacillus pasteurii and from Proteus vulgaris were inactive when assayed at less than pH 5.2 or pH 6.0 respectively, it was observed in the present study that the urease from P. morganii could be subjected to moderately high acidities without permanent loss of enzyme activity. Solutions of both the cell-free extract of P. morganii and of the purified urease preparations could be held at pH 4.6 at room temperature for at least 20 minutes with no apparent loss of ureolytic activity (Table 1). These results suggested that if any loss of urease activity occurred during the purification procedure, it would likely be caused by some factor other than the acidity of the elution buffer. As an added precaution, however, β-mercaptoethanol ($10^{-3}$ M) was included in the buffers which were used

TABLE I

EFFECT OF pH ON ENZYME ACTIVITY OF UREASE FROM *P. MORGANII*

| Enzyme Preparation | Incubation Buffer (Containing $10^{-3}$ M β-mercaptoethanol) | Specific activity after 20 minutes at room temperature (I.U./mg protein) |
|---|---|---|
| Cell-free extract | 0.02 M phosphate buffer, pH 7.0 | 16.12 |
| | 0.02 M phosphate buffer, pH 4.6 | 16.40 |
| Purified urease* | 0.20 M phosphate buffer, pH 7.0 | 2420 |
| | 0.20 M phosphate buffer, pH 4.6 | 2482 |

Samples of cell-free extract and of purified urease were diluted with 9 volumes of buffer, as indicated in the Table, incubated at room temperature for 20 minutes and then assayed for urease activity at pH 7.0 as described in Materials and Methods. Purified urease was diluted with the appropriate buffers as soon as it was eluted from the column. Protein was determined by the method of Lowry *et al.* (1951).

* Urease purified by affinity chromatography.

for washing the column after adsorption of urease and for eluting bound urease from the column, since it had been reported earlier that β-mercaptoethanol stabilized urease activity in aqueous solutions (Magana-Plaza, personal communication; Varner, 1958; Lynn, 1967; and Mamiya and Gorin, 1965).

Inidially, EDTA ($10^{-3}$ M) was also included in the wash and elution buffers in order to protect against a possible inactivation of the urease by divalent cations (Magana-Plaza et al., 1971; Magana-Plaza, personal communication; and Lynn, 1967). It was observed, however, that when $10^{-3}$ M EDTA was included in the elution buffer, a persistent absorbance at 280 nm was observed in fractions collected after the urease-containing, second peak had been completely eluted from the column (Fig. 6). This absorbance at 280 nm was still observed to be present in the effluent at an undiminished level even after a volume of elution buffer equivalent to 25 times the volume of the packed gel had been passed through the gel column (Fig. 6). If the gel column was then washed with distilled water, the absorbance at 280 nm in subsequent fractions disappeared (Fig. 6). However, when the EDTA-containing buffer was again passed through the column, the absorbance of the effluent at 280 nm rapidly increased to the level observed prior to the water wash (Fig. 6). Further investigations revealed that the unidentified component (or components) in these fractions had no ureolytic activity, that they were dialyzable and that they gave no reaction in the Lowry test for protein (Table II).

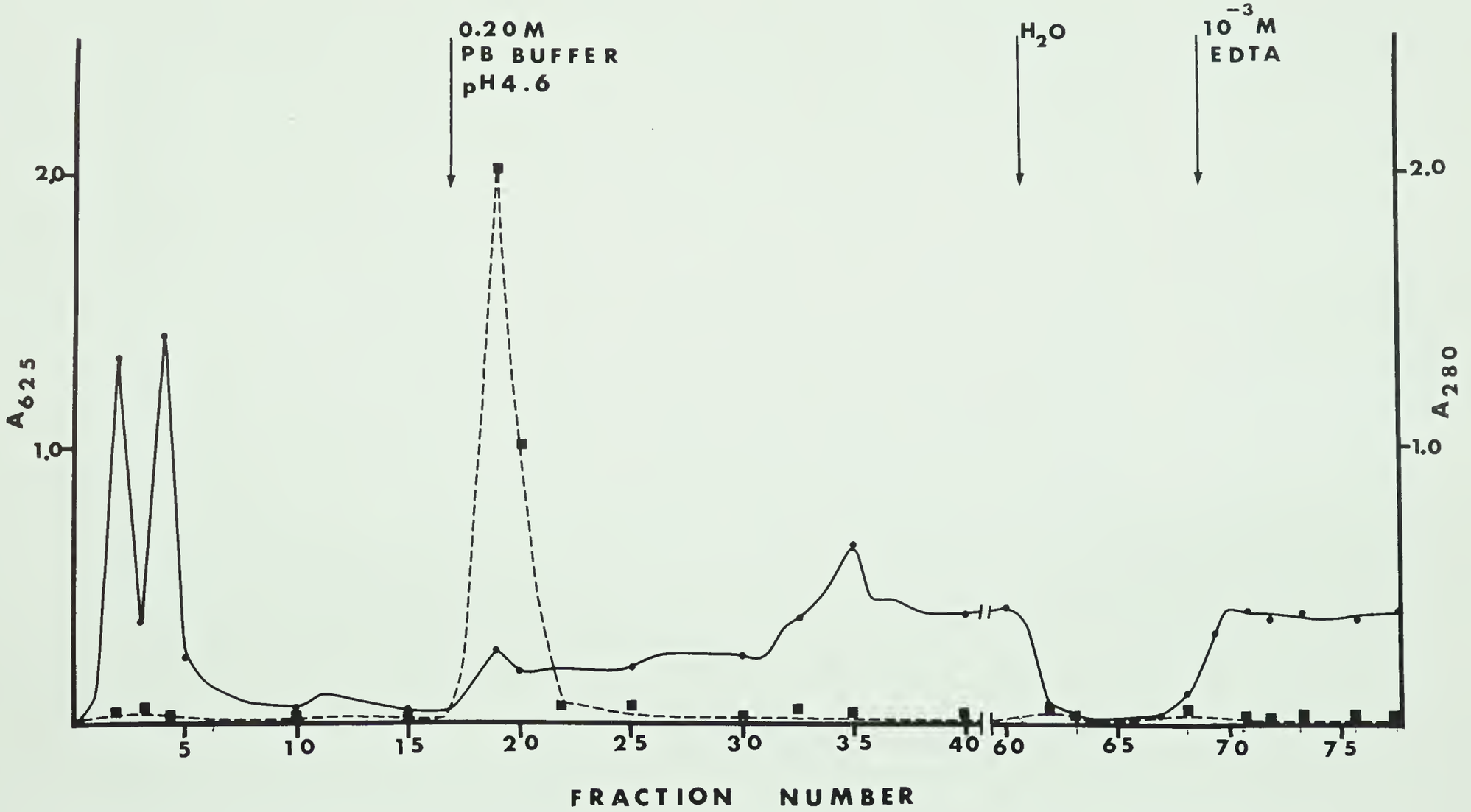
0.20 M
PB BUFFER
pH 4.6
$H_2O$
$10^{-3}$ M
EDTA
2.0
1.0
$A_{625}$
$A_{280}$
5
10
15
20
25
30
35
40
60
65
70
75
FRACTION NUMBER

FIG. 6. Effect of EDTA on absorbance at 280 nm of column effluent.

1.0 ml of dialyzed cell-free extract was chromatographed on a hydroxyurea-Affinose column as described previously. After elution with 0.20 M PB buffer, pH 4.6, containing $10^{-3}$ M EDTA and $10^{-3}$ M β-mercaptoethanol, the column was washed with distilled water and then eluted again with $10^{-3}$ M EDTA. 3-ml fractions were collected and protein was expressed as the absorbance at 280 nm and enzyme activity as the change in absorbance at 625 nm produced by 10 λ of the fraction in the assay system under the conditions specified in Materials and Methods.

Absorbance at 280 nm. ——●——

Change in absorbance at 625 nm. ■----------■

TABLE II

CHARACTERISTICS OF THE UNKNOWN COMPONENT(S) ELUTED FROM THE AFFINITY COLUMN BY $10^{-3}$ M EDTA-CONTAINING BUFFER

| Tube Numbers (1) | Urease Activity (IU/mg Protein) | Absorbance at 280 nm | | Protein Concentration (µg/ml) |
|---|---|---|---|---|
| | | Before Dialysis | After Dialysis (4) | |
| 17 to 22 (2) | 1160 | 0.1675 | 0.1439 | 53.5 |
| 30 to 34 (3) | < 0.01 | 0.1579 | 0.021 | < 1.8 |

(1) The column effluent was collected in 3 ml fractions. For the purpose of this experiment, the contents of the designated tubes were pooled prior to analysis.

(2) Tubes numbered 17 to 22 contained the major peak of eluted urease activity.

(3) Tubes numbered 30 to 34 contained material eluted by EDTA-containing buffer after the major peak of urease activity.

(4) Pooled samples were dialyzed at 0°C for 24 hours against 2 x 1000 volumes of distilled water.

That the EDTA in the elution buffer was responsible for the persistent absorbance at 280 nm could be shown by passing buffers with varying compositions through the same column. The column was washed with several volumes of distilled water between successive changes of buffer and the mean absorbance at 280 nm was determined for ten fractions collected after the column was equilibrated with the different buffering systems. The results of these experiments, outlined in Table III, indicate that while including $10^{-3}$M β-mercaptoethanol in the elution buffer did not significantly elevate the absorbance at 280 nm of the eluted fractions above that observed for elution buffer alone, the addition of EDTA ($10^{-3}$ M) to the buffer did cause a marked increase in the absorbance of the eluted fractions.

In order to determine whether EDTA was required in the elution buffer to stabilize the purified urease derived from P. morganii, 1.0 ml of the cell-free extract in 0.02 M PEB buffer pH 7.0 was dialyzed against a total of 8 liters of distilled water at 0°C for 3 hours in order to remove from the cell-free extract the EDTA and the β-mercapto-ethanol present in the extraction buffer. Aliquots of the dialyzed cell-free extract were then diluted (1:1) with 0.20 M phosphate buffer, pH 4.6, and with the same buffer containing either $10^{-3}$ M EDTA, or $10^{-3}$ M β-mercaptoethanol, or both of these compounds. The urease activity in these preparations was then determined both immediately and after storage of the solutions at 0°C for 24 hours. The results of these experiments (Table IV) indicated that there was no inactivation of the urease from P. morganii after storage for 24 hours at 0°C in buffers

TABLE III

EFFECT OF EDTA ON THE ABSORBANCE AT 280 nm OF FRACTIONS ELUTED FROM THE AFFINITY COLUMN

| Composition of Eluting Buffer | Absorbance* at 280 nm |
|---|---|
| 0.20 phosphate buffer, pH 4.6 | 0.023 |
| 0.20 phosphate buffer, pH 4.6 + $10^{-3}$ M β-mercaptoethanol | 0.037 |
| 0.20 M phosphate buffer, pH 4.6 + $10^{-3}$ M β-mercaptoethanol + $10^{-3}$ M EDTA | 0.599 |

* Mean absorbance of 10 fractions collected after equilibration of the column with the respective buffers.

TABLE IV

EFFECT OF EDTA AND/OR β-MERCAPTOETHANOL ON THE ACTIVITY OF UREASE IN CELL-FREE EXTRACT[1]

| Buffer | Enzyme Activity (IU/ml) | Enzyme Activity after standing at 0°C for 24 hours (IU/ml) | Per Cent Activity Change |
|---|---|---|---|
| 0.10 M phosphate buffer, pH 4.6 | 4.89 | 5.32 | + 8.79 |
| 0.10 M phosphate buffer, pH 4.6 + $10^{-3}$ EDTA | 4.98 | 5.17 | + 3.8 |
| 0.10 M phosphate buffer, pH 4.6 + $10^{-3}$ M β-mercaptoethanol | 4.88 | 5.01 | + 2.4 |
| 0.10 M phosphate buffer, pH 4.6 + $10^{-3}$ M β-mercaptoethanol + $10^{-3}$ M EDTA | 5.16 | 5.60 | + 8.5 |

1. Dialyzed cell-free extract was diluted 1:1 with 0.20 M phosphate buffer, pH 4.6, in the absence or presence of $10^{-3}$ M EDTA and/or $10^{-3}$ M β-mercaptoethanol. Urease activity was determined at pH 7.0, as described in Materials and Methods, both immediately and after incubation in the appropriate buffers at 0°C for 24 hours.

which did not contain EDTA. Since the dialysis step in this procedure may be considered to be analogous to the washing of the column, after the adsorption of urease to the ligand-matrix complex, with a buffer from which EDTA had been omitted, it was concluded that it was not necessary to include EDTA in either the wash or the elution buffers.

Regeneration of the affinity column

When elution of urease activity with 0.20M PB buffer, pH 4.6, was completed, as determined by direct assay of the eluted fractions for urease activity, the column was regenerated by washing the gel with 0.40 M $KH_2PO_4$, pH 4.6, containing $10^{-3}$ M β-mercaptoethanol. This procedure resulted in the elution of an additional component from the column which had a strong absorbance at 280 nm (Fig. 7). No urease activity could be detected in these fractions, however, and when they were pooled and dialyzed at 0°C overnight, and assayed for protein by the Lowry method, little or no protein could be detected (Table V). Approximately 36 per cent of the absorbance at 280 nm was lost during the dialysis procedure, indicating that this material was only partially dialyzable, in contrast with the material eluted from the column by $10^{-3}$ M EDTA, as described above, which was completely dialyzable. The reduction in the absorbance at 280 nm after dialysis was due in part to dilution during dialysis. However, since the material was essentially unreactive with the Folin-Lowry reagent used for protein determination, it was suggested that the material was most likely a low molecular weight polymer such as polypeptide or perhaps a

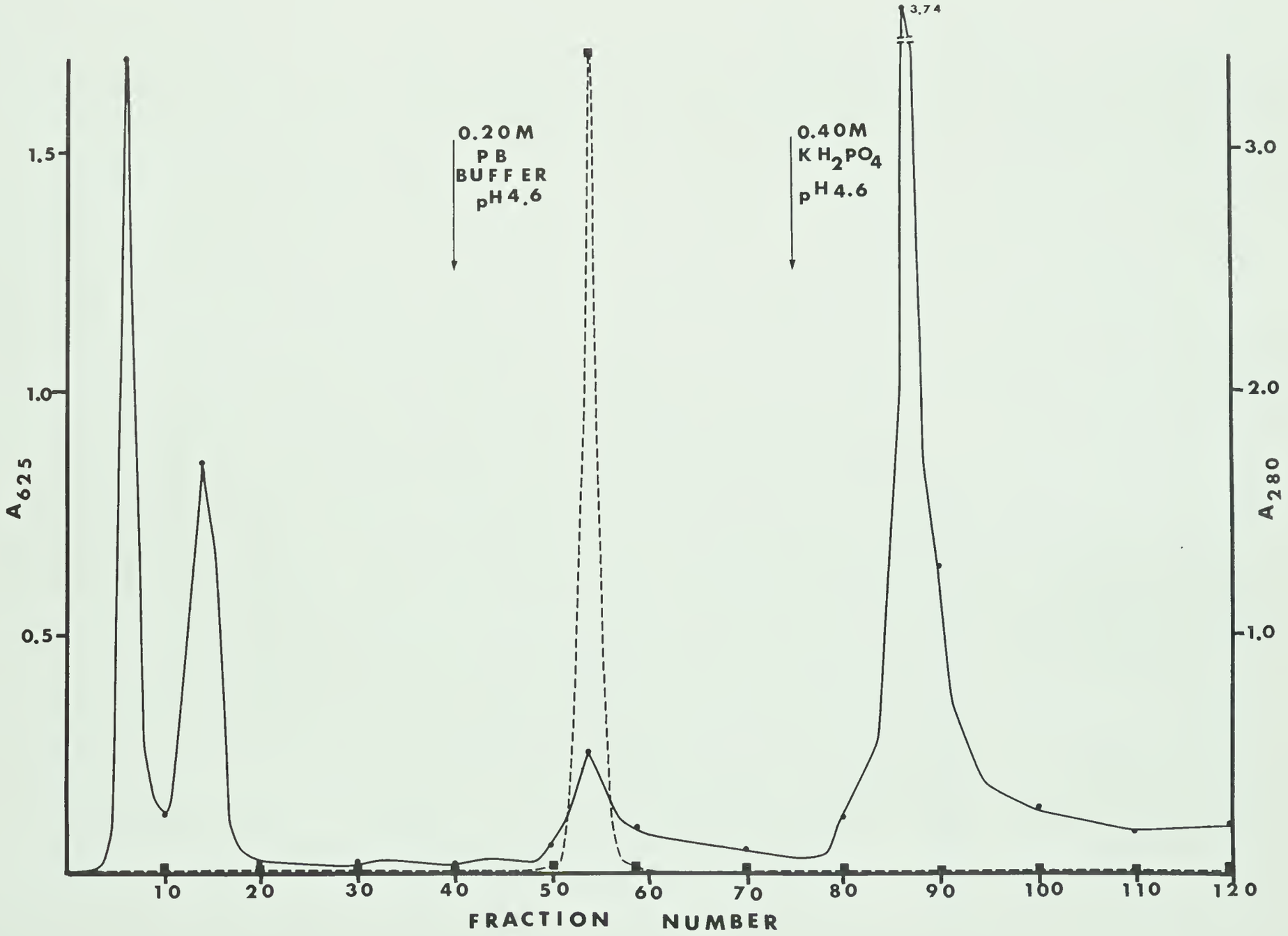
3.74
0.20M
PB
BUFFER
pH4.6
0.40M
KH2PO4
pH4.6
1.5
1.0
0.5
A625
3.0
2.0
1.0
A280
10
20
30
40
50
60
70
80
90
100
110
120
FRACTION NUMBER

FIG. 7. Affinity chromatography of urease from *P. morganii*.

2.0 ml cell-free extract in 0.02 M PB buffer were chromatographed at a constant flow rate of 50 ml/hr on a 1.5 x 6.0 cm hydroxyurea-Affinose column as previously described. After elution of bound urease with 0.20 M PB buffer, pH 4.6, the column was washed with 0.40 M $KH_2PO_4$ pH 4.6 containing $10^{-3}$ M β-mercaptoethanol.

3.3-ml fractions were collected. Protein was expressed as absorbance at 280 nm and urease activity as the change in absorbance at 625 nm produced by 5 λ of the sample in the assay mixture under the conditions specified in Materials and Methods.

Absorbance at 280 nm. ——●——●——

Change in absorbance at 625 nm. ■- - - - - - - - - -■

TABLE V

ANALYSIS OF ELUTED FRACTIONS WITH HIGH ABSORBANCE AT 280 nm

| Tube Number[1] | Absorbance at 280 nm | | Enzyme Activity (IU/mg protein) | Protein Concentration (μg/ml) |
|---|---|---|---|---|
| | Before Dialysis | After Dialysis | | |
| 5 - 18[2] | 0.9469 | 0.6383 | < 0.04 | 100 |
| 51 - 58[3] | 0.3188 | 0.2959 | 1378 | 59 |
| 80 - 100[4] | 0.9872 | 0.6364 | < 0.01 | 10 |

(1) The column effluent was collected in 3.3 ml fractions. For the purpose of this experiment, the designated fractions were pooled prior to dialysis.

(2) Fractions containing material which was not adsorbed to the affinity column.

(3) Fractions containing the major peak of eluted urease activity.

(4) Fractions eluted with 0.40M $KH_2PO_4$, pH 4.6, during regeneration of the column.

polynucleotide.

There was some concern initially that this material was released from the column as a result of a degeneration of the ligand-matrix complex which might have been induced either by the buffers employed for washing, eluting and regenerating the gel, or as a result of the activity of one or more of the enzymes present in the cell-free extract. Continued use of the same column, however, led to the conclusion that no significant degeneration of the ligand-matrix complex was occurring, since after 20 successive experiments in which the above-outlined adsorption, washing, elution and regeneration sequence was carried out, the capacity of the column for the adsorption of urease was reduced by less than 5 per cent. It was also observed that when jack bean urease was chromatographed rather than the cell-free extract of P. morganii, no 'third peak' of material with a high absorbance at 280 nm was eluted by the regeneration buffer. This finding supported the earlier suggestion that the eluted substance was derived from the cell-free extract of P. morganii, rather than being a product of the degeneration of the ligand-matrix complex. When dialyzed, lyophilized samples of this 'third peak' component were subjected to polyacrylamide gel electrophoresis and the gels stained for protein and urease activity as described in Materials and Methods, neither protein bands nor bands of urease activity could be detected in these gels (Fig. 8), thus supporting the conclusion reached above that the component was non-protein in nature.

-

a b

+

FIG. 8. Electrophoresis of 'Third Peak' components

Lyophilized, pooled 'third peak' fractions were subjected to polyacrylamide electrophoresis as described in Materials and Methods.

Gel a was stained with Amido black.

Gel b was stained with the catalytic stain.

Distortion of Gel b was the result of rapid shrinkage following the use of the catalytic staining technique.

Temperature of adsorption and elution

When 1.0 ml of dialyzed cell-free extract in 0.02 M PB buffer, pH 7.0, was added to a 1.5 x 6.0 cm column and adsorbed to and eluted from the ligand-matrix complex, using the procedures outlined above, either at room temperature or at 0°C, there were no significant differences between the total amounts of urease activity bound to the columns or the specific activities of the urease preparations eluted from the columns under either of these temperature conditions (Table VI). This observation suggested that the urease enzyme had a high affinity for the bound ligand at pH 7.0, since if the affinity was low, differences might have been observed in the amount of enzyme bound to the column at the lower temperature. These findings provide additional indirect evidence that the bound ligand is functioning as an immobilized long-chain aliphatic hydroxamic acid within the column, since it has been reported that the $K_i$ values for the inhibition of urease by long-chain ($C_2$ to $C_{11}$) aliphatic hydroxamic acids are in the order of 1.2 to $10^{-6}$ M to 9.3 to $10^{-8}$ M (Kobashi et al., 1962). Since it appeared that temperature effects would not alter enzyme recovery or purity, subsequent experiments were carried out at room temperature.

Purification of urease from P. morganii

Having established a technique for the preparation of a ligand-matrix complex which would effectively adsorb the urease present in solutions, and having determined the optimum conditions for adsorption and elution of urease from the affinity column, the direction

TABLE VI

EFFECT OF ELUTION TEMPERATURE ON QUANTITY OF UREASE BOUND TO THE COLUMN

| | Temperature of Adsorption and Elution | |
|---|---|---|
| | 0°C | 25°C |
| Total protein (μg) | 432 | 431 |
| Total urease activity (IU) | 1127 | 1082 |
| Specific activity (IU/mg protein) | 2613 | 2515 |

1.0 ml of dialyzed cell-free extract in 0.02 M PB buffer was chromatographed on 1.5 x 6.0 cm hydroxyurea-substituted columns, either at 0°C or at room temperature. The enzyme activity and protein concentrations were determined as described in Materials and Methods.

of this investigation was turned toward the purification of urease from P. morganii. When 1.0 ml of dialyzed cell-free extract of P. morganii in 0.02 M PB buffer, pH 7.0 was passed through a 1.5 x 6.0 cm column of hydroxyurea-substituted Affinose AF-202, no urease activity could be detected in the first protein peak to emerge from the column. Nor was any urease activity eluted from the column when the gel was subsequently washed with 0.02 M PB buffer, pH 7.0 (Fig. 9). However, when 0.20M PB buffer, pH 4.6, was passed through the column, a small protein peak was eluted which was shown to contain a very high urease activity (Fig. 9). Comparison of the specific activity of the urease from this second peak with that of the urease in the cell-free extract demonstrated that a 170-fold purification of urease had been achieved by this simple chromatographic procedure (Table VII, No. 4). In a number of similar experiments, single-step purifications of between 126 and 231 times have been achieved. It has been suggested that the variations in the degree of purification of urease achieved by this technique are likely a reflection of the inherent instability of purified preparations of this enzyme and that the lower specific activities of some of the preparations probably resulted from the presence of inactive urease molecules rather than from the presence of contaminating proteins (Larson and Kallio, 1954; Lynn, 1967; Magana-Plaza, personal communication; Magana-Plaza et al., 1971; and Fishbein, 1969). The pooled, dialyzed, urease-containing fractions which were eluted from the column contained, on an average, about 4.6 per cent of the total protein which was present in the cell-free extract introduced into the columns,

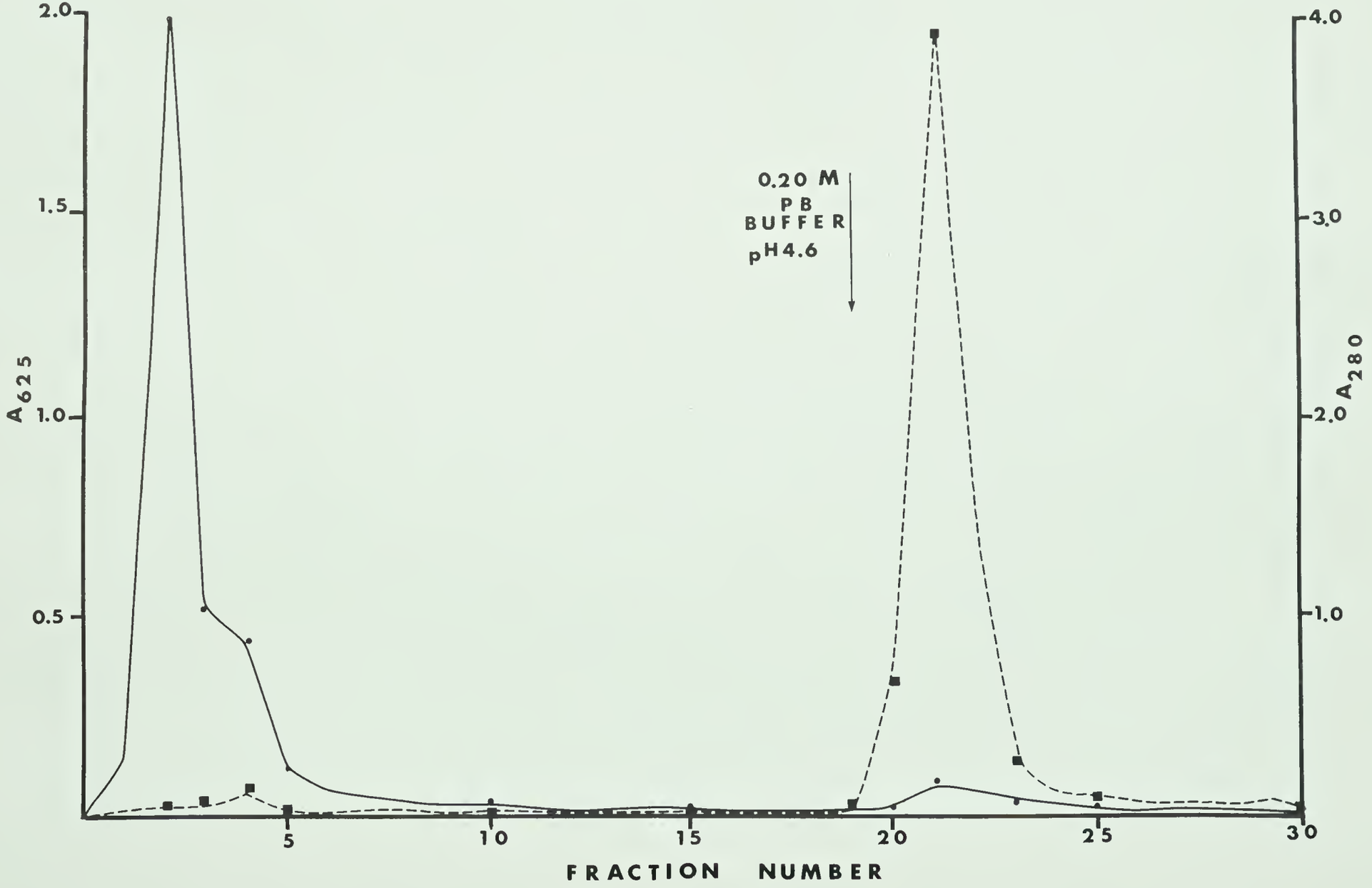
2.0
1.5
1.0
0.5
$A_{625}$
4.0
3.0
2.0
1.0
$A_{280}$
0.20 M
PB
BUFFER
pH4.6
5
10
15
20
25
30
FRACTION NUMBER

FIG. 9. Purification of urease of P. morganii.

1.0 ml of cell-free extract in 0.02 M PB buffer was introduced into a 1.5 x 6.0 cm Hydroxyurea-substituted affinity column and chromatographed under a constant flow rate of 50 ml/hr as described in Materials and Methods. 3.3-ml fractions were collected. Protein was expressed as absorbance at 280 nm and urease activity as the change in absorbance at 625 nm produced by 10 λ of the fraction in the assay mixture under the conditions specified in Materials and Methods.

Absorbance at 280 nm. 

Change in absorbance at 625 nm. 

TABLE VII

PURIFICATION OF UREASE BY AFFINITY CHROMATOGRAPHY

| Prepa-ration Number | Specific Activity in cell-free Extract (IU/mg protein) | Total Activity in cell-free Extract | Specific Activity of Purified Urease (IU/mg Protein) | Total Activity Recovered | Purifi-cation | Per Cent Recovery |
|---|---|---|---|---|---|---|
| 1 | 15.74 | 98 | 2613 | 210.1 | 166 | 214 |
| 2 | 13.73 | 250 | 1882 | 685.0 | 137 | 265 |
| 3 | 13.26 | 122 | 2428 | 240.0 | 183 | 196 |
| 4 | 14.75 | 118 | 2516 | 262.0 | 170 | 230 |
| 5 | 14.04 | 69 | 3243 | 138.7 | 231 | 200 |
| 6 | 15.12 | 63 | 1905 | 143.2 | 126 | 227 |

Cell-free extract of P. morganii was chromatographed on hydroxyurea-substituted Affinose columns as described in the text. The specific activity of the purified urease was that of the peak fraction in each run.

indicating that urease is one of the major proteins present in P. morganii. In addition, the observation that the recovery of urease activity was always greater than 200 per cent (Table VII) suggested that activation of the enzyme occurred during the purification procedure.

In general, a complete chromatographic cycle, involving the adsorption of urease, the washing of the gel and the elution of urease, followed by the regeneration of the gel and re-equilibration of the column in preparation for the next purification cycle, could be carried out in less than 90 minutes. Since the ligand-matrix complex was specifically designed so that it would selectively adsorb only urease and no other proteins, in contrast with ion exchange resins, gel columns with small dimensions but large capacities for adsorbing urease could be employed. As a result, small volumes of the various buffers, as well as flow rates in the order of 100 - 200 ml/hr could be used during the chromatographic procedure with no adverse effects either on column stability or on column resolution. These factors were considered to be directly related to the short cycling time of these columns.

Polyacrylamide (disc) gel electrophoresis

Cell-free extract, pooled samples of the unadsorbed proteins and pooled samples of the purified urease (second protein peak) were dialyzed at 0°C against 2 x 100 volumes of $10^{-3}$ M β-mercaptoethanol and lyophilized in a New Brunswick Cryolizer, Model B65. The lyophilized samples were then redissolved in a minimal volume of

distilled water. Portions of these solutions, containing between 50 µg and 200 µg of protein, were diluted with equal volumes of 40 per cent sucrose and the resulting solutions were layered onto the spacer gels in the gel cylinders in preparation for electrophoresis. Duplicates were prepared for each sample. Spacer gels and separating gels were prepared as described in Materials and Methods. Electrophoresis of the samples was carried out in 0.05 M Tris-glycine buffer, pH 8.8, at 0°C for 4 hours, using a current of 2 mA per gel cylinder.

After 4 hours, the gels were removed from the gel cylinders and duplicate gels were stained either with Amido black, or with the catalytic stain for urease described in Materials and Methods.

A large number of protein bands (as shown by the Amido-black stain) were observed in the gel to which the cell-free extract had been added (Fig. 10a), while a duplicate gel, stained with the catalytic urease stain showed only a single band of urease activity (Fig. 10b). As can be seen, this band of urease activity corresponded to one of the slow-migrating protein bands. In the gels to which samples of the unadsorbed protein peak had been added, although numerous bands were again observed (Fig. 11a), no catalytically stained bands were detected (Fig. 11b), indicating that there was no assayable urease in the unadsorbed protein peak fractions. Since the catalytic stain has been reported by Fishbein (1969) to be a more sensitive technique for the detection of urease activity than any other assay procedure, this observation confirmed the similar earlier conclusions which had been based upon the routine assay for urease activity that is described in

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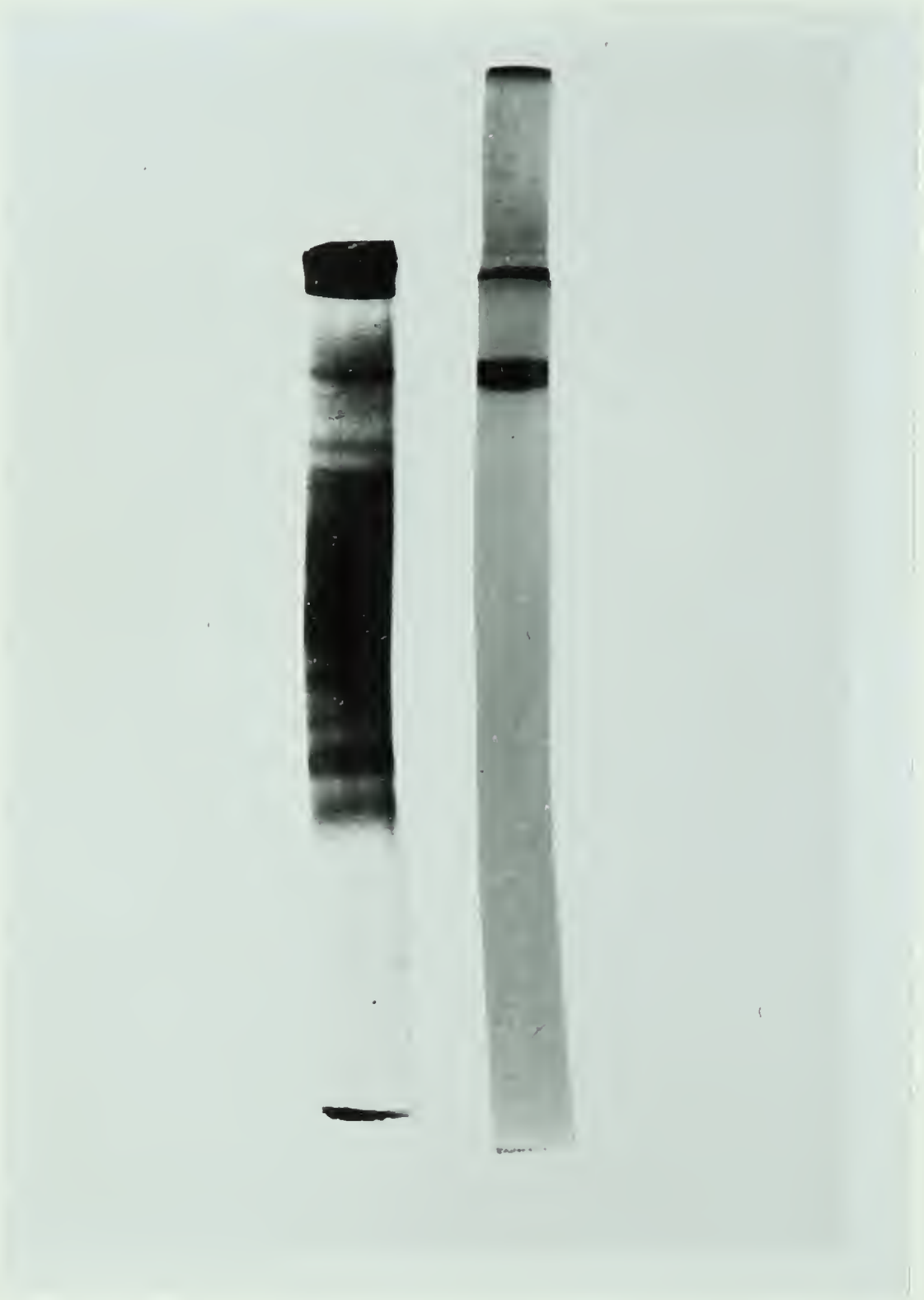

a b

+

FIG. 10. Electrophoresis of cell-free extract.

Lyophilized cell-free extract containing 200 μg of protein was subjected to polyacrylamide gel electrophoresis, in duplicate, as described in Materials and Methods.

Gel a was stained with Amido black.

Gel b was stained with the catalytic stain.

-

a b

+

FIG. 11. Electrophoresis of unadsorbed 'First Peak' components.

Lyophilized, pooled 'first-peak' fractions containing 130 μg of protein were subjected to polyacrylamide gel electrophoresis as described in Materials and Methods.

Gel a was stained with Amido black.

Gel b was stained with the catalytic stain.

Materials and Methods. In the gels to which had been added samples of the urease-containing second protein peak, 12 protein bands were discernable with the Amido-black stain (Fig. 12a) and all but four of these bands corresponded to areas of the gel which contained urease activity as demonstrated by the catalytic stain (Fig. 12b). As a control, a duplicate gel was reacted with the catalytic staining reagents from which urea had been omitted. As is shown in Fig. 13, no bands developed in this gel in the absence of urea. This observation suggests that those bands which were catalytically stained by this technique probably represent urease isozymes or catalytically active subunits of urease analogous to those reported by Fishbein (1969). The protein bands which do not correspond to ureolytic areas of the gel may represent either inactive isozymes or catalytically inactive subunits of the urease enzyme, or they may represent contaminating proteins which also possess an affinity for the hydroxamic-acid ligand. Evidence from other sources (discussed below) tends to favor the hypothesis that the catalytically inactive bands in these gels represent inactive subunits of urease.

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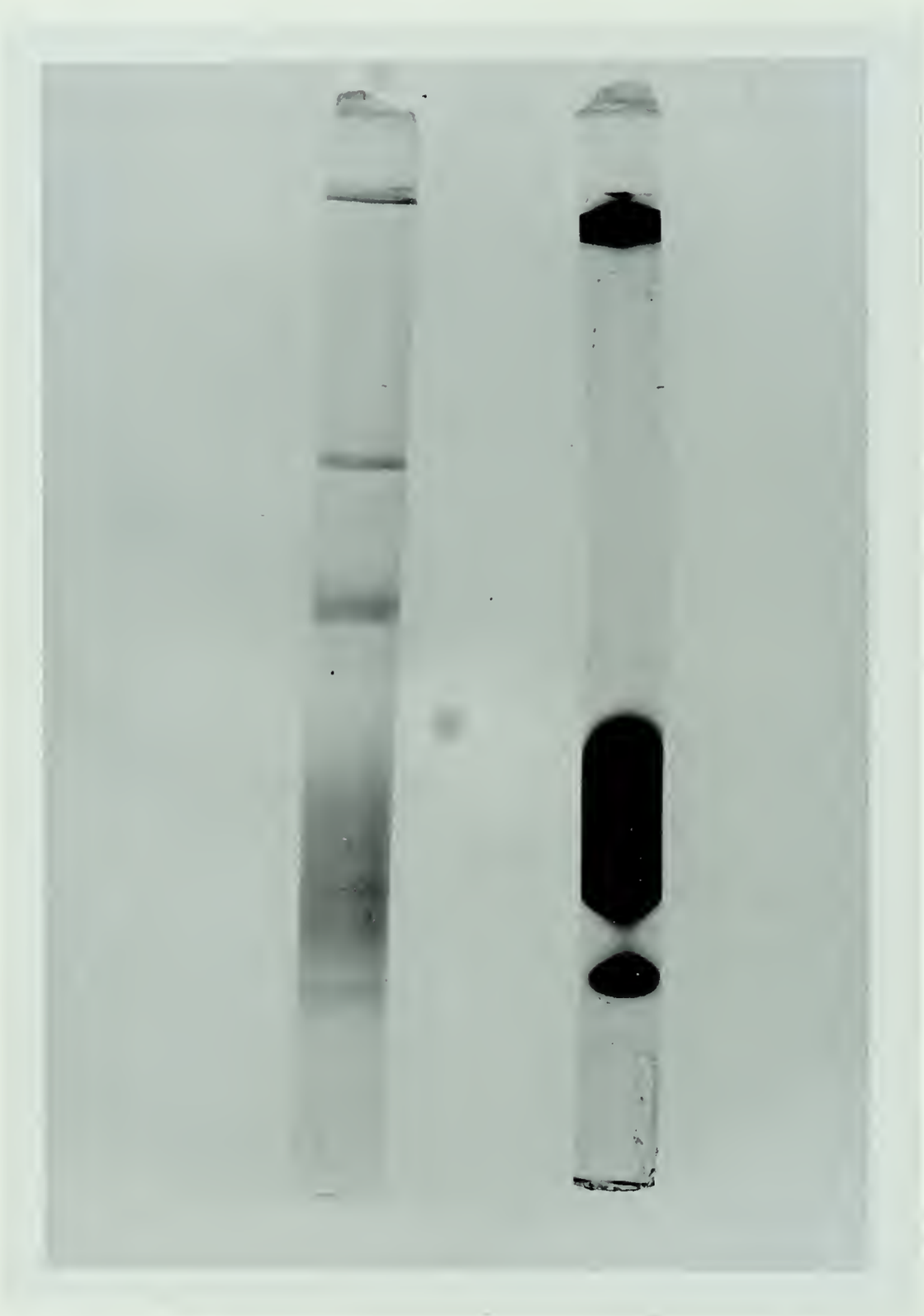


a b

+

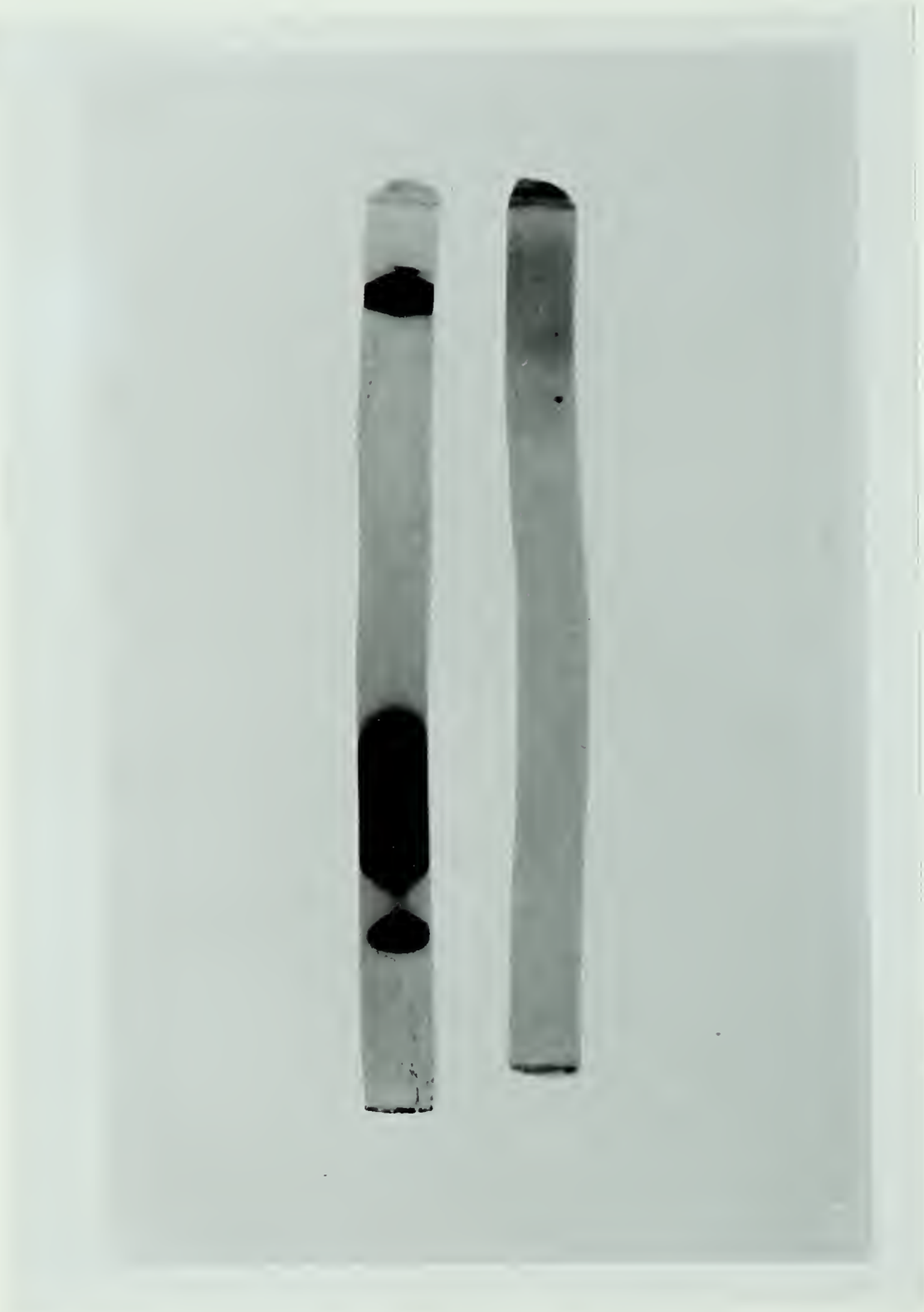
FIG. 12. Electrophoresis of purified urease.

Samples of lyophilized, pooled urease-containing fractions containing 100 μg of protein were subjected to polyacrylamide gel electrophoresis, as described in Materials and Methods.

Gel a was stained with Amido black.

Gel b was stained with the catalytic stain.

-



a b

+

FIG. 13. Specificity of catalytic stain.

Lyophilized, pooled urease-containing fractions containing 100 µg of protein were subjected to polyacrylamide gel electrophoresis as described in Materials and Methods. Gel a was stained with the complete catalytic stain. Gel b was stained with the catalytic stain from which urea was omitted.

## DISCUSSION

Although the reaction mechanism for the 1-Ethyl-3-(3-dimethyl-aminopropyl) carbodiimide-mediated binding of hydroxyurea to the Affinose AF-202 beads is not well understood, there was evidence to suggest that the reaction resulted in the formation of a ligand similar to a long-chained aliphatic hydroxamic acid, as was outlined in Fig. 1. The rise in pH as the reaction proceeded may have reflected the formation of an amidoxime, a group of organic chemicals which include the hydroxamic acids, since it was reported by Eloy and Lenaers (1962) that amidoximes have basic properties. As well, the appearance of a persistent orange color when the reacted gel was treated with the TNBS-borate reagent indicated that substitution of the free carboxyl groups of the Affinose gel was occurring. Since free amino groups will react readily with free carboxyl groups under the influence of the carbodiimides, the most likely result of the reaction, in this instance, would be the formation of a terminal hydroxamic acid group on the side chain of the Affinose gel.

That a hydroxamic acid ligand was formed was also indicated by the observation, during the subsequent use of the substituted gel, that urease had a strong affinity for the ligand, as was indicated by the fact that there were no differences between the extent of binding of urease to the gel at 0°C and at 20°C. It has been suggested that ureases may have very high affinities for hydroxamic acids, since $K_i$ values in the order of $10^{-7}$ M to $10^{-8}$ M have been reported for the

aliphatic hydroxamic acids. These observations, therefore, also suggest that the ligand which was formed by the binding of hydroxyurea to the Affinose AF-202 gel under the conditions employed may be an analogue of a long-chained hydroxamic acid.

During the preparation of the ligand-matrix complex, the degree of substitution of the side chain with hydroxyurea was monitored with the TNBS-borate color test. Although a strong positive color reaction always indicated that an extensive substitution of the gel was occurring, a quantitative assessment of the degree of substitution could not be made in this way because the color reaction varied considerably from gel preparation to gel preparation, both in the rate of color development and in the final color intensity. An accurate assessment of the degree of substitution of the gel by the ligand could be achieved only by measuring the amount of urease activity which was retained by the gel. Alternative methods for determining the degree of substitution of the gel could have included acid hydrolysis of the gel followed by quantitative estimation of soluble hydroxamic acid residues in the hydrolysate, or the use of radioactive ligand so that the amount of ligand bound to the gel following the substitution reaction could be determined by scintillation-counting methods. It is also theoretically possible to determine the degree of substitution of the gel by quantitating the ligand which remained unbound at the end of the reaction interval. This method would not be suitable in the present instance, since it was necessary to wash the reacted gel with large volumes of buffer (600 ml/ml of

reacted gel) in order to remove completely the residual unbound hydroxyurea, and the problems involved in concentrating the wash buffer down to an assayable volume in order to accurately determine the amount of residual unbound hydroxyurea would have been considerable. Since column capacity could easily be determined by directly measuring the amount of adsorbed urease activity, this procedure would not have provided any additional useful information.

When the TNBS-borate color reaction of hydroxyurea-substituted Affinose was compared with that of hydroxyurea-substituted succinyl-amino alkyl Sepharose 4-B, it was observed that the color developed much more rapidly and to a much higher intensity in the Sepharose gel. The difference was so striking that it suggested that the Sepharose gel was much more extensively substituted with hydroxyurea than was the Affinose gel, even though it had been found that the TNBS-borate color test was not a reliable quantitative test. This hypothesis was also suggested by the observation that larger quantities of protein could be adsorbed by the Sepharose gel in comparison to the amounts adsorbed by the substituted Affinose gel. Unfortunately, active urease preparations could not be eluted from the Sepharose column. Although those columns appeared to possess a very high capacity for binding urease and therefore could have been used to purify much larger quantities of urease in each experiment than was possible with similar sized columns of the substituted Affinose gel, the problem of eluting an active urease preparation from the substituted Sepharose columns could not be solved.

Other reports in the literature suggest that columns prepared

from Sepharose or beaded agarose usually possess very high capacities for binding protein (Cuatrecasas et al., 1968; Steers et al., 1971). This does not appear to be true for the substituted Affinose AF-202 gels, since only microgram amounts of urease could be prepared in each experiment using these columns. Using columns with similar bed volumes, but alternative carriers to support the ligands, other investigators have reported the purification of milligram amounts of other enzymes (Cuatrecasas et al., 1968; Steers et al., 1971). Although the use proved valuable within the context of this investigation, the use of Affinose gels by other investigators is not recommended because of the apparent low binding capacity. It is further recommended that the investigation into the use of substituted Sepharose columns for the purification of urease should be continued.

Although the capacity of the substituted Affinose gel for the adsorption of urease was small, significant quantities of enzyme could be purified within a relatively short time because of the rapid cycling possible with the column. Little, if any, degeneration of the ligand-matrix complex occurred even after prolonged use, so that there were no problems involved in frequent use of the same gel column. As well, since only simple washing of the used gel with an appropriate buffer was necessary in order to regenerate the gel, the column could be re-used for the application of a new sample very rapidly. As a result, it was possible to purify milligram quantities of urease within a single day. In addition, ureases from more than one source could be purified on the same ligand-matrix complex.

The specific activities of the preparations of purified urease which were obtained from P. morganii cell-free extracts by this technique were consistently in the order of 2000 to 2500 I.U./mg of protein. These values are 1.2 to 1.5 times higher than those reported by Larson and Kallio (1953) for the purified urease they obtained in 9 per cent yields by selective precipitation of cell-free extracts of Bacillus pasteurii. The specific activity of the crystalline urease prepared from P. rettgeri by Magana-Plaza et al. (1971; and personal communication) was approximately 31 I.U./mg of protein. Besides, the yield of enzyme was only 2 per cent, while in the investigation reported here, the yields of enzyme are consistently greater than 200 per cent. The specific activities of crystalline urease preparations obtained from jack bean meal have also been in the order of 1000 to 2000 I.U./mg of protein, but again, the recovery of enzyme has been consistently low (Fishbein, 1969; Reithel and Robbins, 1967; Mamiya and Gorin, 1965; and Lynn, 1967).

The only other instance in which greater than 200 per cent recovery of urease activity has been achieved was reported by Fishbein (1969), who subjected purified crystalline urease from jack bean to further recrystallization and gel fractionation procedures. The increase in the enzymatic activity of urease preparations following this procedure was attributed to the release, from a large urease aggregate, of subunits which had higher specific activities than did the original urease aggregate (Fishbein, 1969).

An analogous process of enzyme activation appears to occur

when the urease from P. morganii is purified on hydroxyurea-substituted Affinose columns, since a similar dissociation of a large urease aggregate occurs either during or subsequent to the elution of the purified enzyme from the affinity column. This suggestion is supported by the observation of numerous bands of urease activity in the catalytically stained polyacrylamide gels obtained by disc gel electrophoresis of the purified urease preparation, in contrast to the single band of urease activity which was found in polyacrylamide gels to which had been applied samples of the cell-free extract of P. morganii.

The appearance of a few catalytically inactive protein bands in the polyacrylamide gels prepared using the purified urease preparation does not necessarily indicate that proteins other than urease were being adsorbed to the affinity column. Alternative suggestions include the possibility of limited proteolysis of urease, either in the cell-free extract or while urease was bound to the ligand during the column-washing procedure, and the possibility that catalytically inactive subunits of the urease aggregate are released as a result of the dissociation of the enzyme which has been shown to occur. This latter hypothesis is supported by the earlier report by Fishbein (1969) that catalytically inactive subunits were released from preparations of crystalline jack bean urease which were subjected to further fractionation procedures. However, one cannot at this time exclude the possibility that enzymes other than urease may have a significant affinity for the hydroxamic acid ligand and a further

investigation into the identity of the catalytically inactive proteins is warranted.

That the ureases from both P. morganii and from jack bean meal can be purified on this hydroxamic acid-substituted affinity column suggests that it may also be possible to purify ureases from other sources using the same ligand-matrix complex. This finding also suggests that as a general principle, the ligands of affinity columns may exhibit "substrate" specificity, but not species specificity. In other words, it may be possible to design ligand-matrix complexes which can be used for the purification of those macromolecules which they were designed to adsorb, regardless of the biological source of those macromolecules. Should this prove to be possible, one can readily visualize the advantages of this technique when one considers that it might be necessary to design entirely new purification sequences in order to purify the same enzyme from different sources if classical fractionation techniques were being employed.

# CONCLUSIONS

A rapid, simple, effective method for the purification of the urease from P. morganii by affinity chromatography has been developed. In a single-step procedure, taking less than 90 minutes, a urease preparation with a specific activity as high as, or higher than, any previously reported for purified urease, has been prepared from a crude, cell-free extract, with yields of enzyme that are from 20 to 150 times as great as any previously reported. In addition, the fact that this ligand-matrix complex can be used for the purification of ureases from two very different sources also suggests that it may be useful in the purification of urease from many other sources as well.

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